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Impact of proteolysis on pH decline and water-holding capacity of fresh pork

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General Introduction

Quantity and quality are two important terms in the swine industry. Currently, the marketing system in the United States pays for quantity not quality. In response, producers have continually striven for quantity by raising more muscular, faster growing hogs than those of previous generations. Unfortunately, heavily muscled genetics carry a stigmatism of being poorer in quality (Sonesson, de Greef, Meuwissen, 1998). In addition, as consumers become more discriminating of the products they purchase, the demand for quality has risen above the demand for quantity, thus imparting a need for understanding the biological and biochemical factors that impact the quality aspect.

High quality pork does not have a common definition in the industry today; rather, it can be quantified as a combination of inherent properties such as water-holding capacity, color, flavor, juiciness, tenderness, fat content and composition, oxidative stability and uniformity (Rosenvold and Andersen, 2003). Lack of consistently high quality pork has become a major obstacle standing in the way of increasing pork consumption. The swine industry is at a standstill; the amount of pork consumed per capita in the United States has remained stable from 1970 to 2001 (National Pork Board, 2002/2003). Meanwhile, consumers have increased their consumption of other meat products such as poultry, fish and protein alternatives from plant sources. As a result, all segments of the swine industry, from producers to consumers, have been negatively influenced by poor pork quality. It is important that steps are taken to improve the quality of pork in order for the industry to remain competitive and profitable.

Numerous factors such as genetics, feeding, pre-transport handling, transport, lairage, stunning, postmortem temperature, pH, and chilling can individually or in combination

negatively impact quality attributes (Berg, 1998). Detrimental factors peri- and post-slaughter can create undesirable products such as pale, soft, and exudative pork (PSE); red, soft, and exudative (RSE) pork; or dark, firm, and dry (DFD) pork. Although DFD pork is undesirable due to its dark color and susceptibility to spoilage, most often it is pork which is pale in color, exudes drip, and has an open texture (PSE) that dissuades consumers and causes a loss of sales. Lower yields, increased cooking losses, reduced juiciness and poor processing properties are a direct result of PSE and the high value cuts of the loin and ham are predominately affected. And, not only are there economic losses due to the unappealing drip, but soluble nutrients found in the purge are also lost.

The rate and extent of pH decline has long been known to influence meat color and water holding capacity (Briskey, 1964). A rapid pH decline that results in a low ultimate pH while the muscle is still warm can cause denaturation of proteins that are involved in binding water (Offer and Knight, 1988b). Also, a normal rate of pH decline with a very low ultimate pH (pH 5.0-5.2) negatively influences the water-holding capacity of fresh pork (Offer and Knight, 1988b). Additionally, a high ultimate pH (above 6.0) due to glycogen depletion prior to rigor can negatively result in pork that is dark in color, firm textured, and dry in appearance (DFD) (Offer and Knight, 1998b). Clearly, the factors that control the rate and extent of pH decline need to be researched if the quality issue of poor water-holding capacity is to be solved.

Calcium regulation by the skeletal muscle ryanodine receptor (RyR1) and the sarcoplasmic reticulum Ca^{2+}-ATPase pump (SERCA) postmortem can affect the rate of pH decline. Interestingly, the most severe drip loss is found in PSE products from pigs that have inherited a mutation (halothane gene) in the ryanodine receptor/calcium release channel, a

protein found in the sarcoplasmic reticulum that is responsible for Ca^{2+} release into the sarcoplasm. This mutation results in an impaired ability of the ryanodine receptor to control calcium release into the sarcoplasm of the muscle cell, particularly under periods of stress. An accelerated release of calcium can occur, which causes strong and rapid muscle contraction and an increase in the rate of muscle metabolism and in the rate of pH decline. Despite successful efforts to reduce the occurrence of PSE pork by selectively breeding against the Halothane gene that is the result of a mutation in the ryanodine receptor, there is still immense variability in water-holding capacity (Purslow et al., 2001). This fact suggests that other biochemical factors in muscle need to be examined. As calcium is an important mediator of muscle metabolism, it is highly likely that other alterations in calcium regulation may explain some of the remaining variations in the rate of pH decline.

The sarcoplasmic reticulum Ca^{2+}-ATPase pump (SERCA) is also involved in calcium regulation by removing calcium from the sarcoplasm and returning it to the sarcoplasmic reticulum. The SERCA pump is ATP-dependent and functions as long as ATP is available. Failure of the SERCA to remove Ca^{2+} from the sarcoplasm and return it to the sarcoplasmic reticulum, particularly during the early postmortem period, could result in an increased metabolism and a more rapid rate of pH decline.

Two Ca^{2+}dependent cysteine proteases (μ-calpain, m-calpain) and their naturally occurring inhibitor (calpastatin), have a number of different roles in cells including remodeling of cytoskeletal attachments to the plasma membrane during cell fusion and cell motility, proteolytic modification of molecules in signal transduction pathways, degradation of enzymes controlling the cell cycle, regulation of gene expression, substrate degradation in apoptosis, and involvement in long term potentiation (Goll, Thompson, Li, Wei and Cong,

2003). The calpain system has also been shown to have an instrumental role in postmortem proteolysis (Koohmaraie, 1988) and pH can affect the activity of μ- and m-calpain during proteolysis. Rowe, Huff-Lonergan and Lonergan (2001a) found that the rate of pH decline and ultimate pH influences calpain activity and autolysis, thus, affects the rate of proteolysis. Both the ryanodine receptor (Brandt, Caswell, Brandt, Brew and Mellgren, 1992; Iino, Takano-Ohmura, Kawana and Endo, 1992; Shevchenko, Feng, Varsanyi and Shoshan-Barmatz, 1998; Shoshan-Barmatz, Weil, Varsanyi and Heilmeyer, 1994) and the sarcoplasmic reticulum Ca^{2+}-ATPase pump (Purintrapiban, Wang and Fosberg, 2001; Seiler, Wegener, Whang, Hathaway and Jones, 1984; Wang, Roufogalis and Villalobo, 1988a and 1988b; Zhao, Levin, Wein, and Levin, 1997) are substrates of the calpain system. It is possible that degradation of the SERCA pump by calpain would impair its ability to remove calcium from the sarcoplasm (Zhao et al., 1997). The subsequent higher concentration of calcium in the sarcoplasm could thus cause a faster rate of anaerobic metabolism and an accelerated pH decline. Degradation of the ryanodine receptor by calpain would stimulate Ca^{2+} entry into the cell (Iino et al, 1992; Shoshan-Barmatz et al., 1994) and could also result in an accelerated pH decline. The calpain system may also be affected by modulation of calcium regulation by the RyR or SERCA pump. An increase in sarcoplasmic Ca^{2+} could provide enough calcium to activate μ-calpain. Thus, it is important to characterize the relationship between the degradation of the proteins involved in calcium homeostasis and the calpain system.

The objective of the work in this thesis is to determine the relationship between pH decline, μ-calpain autolysis and degradation of membrane proteins involved in calcium homeostasis. Proteolytic cleavage of the ryanodine receptor and the sarcoplasmic reticulum

Ca^{2+}-ATPase pump in addition to autolysis of μ-calpain is expected to be accelerated in samples from pigs with more rapid and/or more extended pH decline. The hypothesis of this project is that proteolytic processes in early postmortem pork influence pH decline and water-holding capacity. Degradation of the ryanodine receptor and SERCA pump by the calpain system would destabilize calcium regulation in early postmortem muscle and may be involved in more rapid pH decline. Additionally, modulation of the SERCA pump and RyR could cause the activation of μ-calpain due to the increased concentration of calcium in the cell sarcoplasm. To complete the project objective and examine the hypothesis, pork loin chops from two separate genetic lines that differ significantly in ultimate pH but that are free of the halothane and RN- gene were sampled. Within the two genetic lines, two groups were divided based on high and low pH measurements. Groups of pigs with longissimus muscles that have relatively high early postmortem and/or ultimate pH and groups of animals that have relatively low early postmortem and/or ultimate pH will be examined in order to allow autolysis of μ-calpain and degradation of the skeletal muscle ryanodine receptor (RyR1), the sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) pump, and the costameric protein vinculin to be studied at these conditions.

Thesis Organization

This thesis is in an alternate style format consisting of a general introduction, a review of literature, a paper prepared for publication, and a concluding summary. The paper represents the work done by the first author to fulfill requirements for the degree of Master of Science. The paper was prepared according to the Meat Science Style and Form guide. This

paper consists of an Abstract, Introduction, Materials and Methods, Results and Discussion, and Literature Cited sections.

Introduction to Literature Review

The focus of this thesis was to explain some of the biophysical/biochemical mechanisms that influence water-holding capacity in fresh pork. The overall objective of this project was to examine the relationship between pH decline, μ-calpain autolysis and degradation of membrane proteins involved in calcium homeostasis. The central hypothesis was that proteolytic processes in early postmortem pork influence pH decline and water-holding capacity. The following topics are relevant to this hypothesis and will be discussed in this literature review.

I. Water-holding Capacity

II. Overview of the Structure of Skeletal Muscle

III. Muscle Contraction/Relaxation and Calcium Signaling

IV. Muscle Metabolism and Rigor Mortis

V. The Sarcoplasmic Reticulum Ca^{2+}-ATPase (SERCA) pump

VI. The Ryanodine Receptor (RyR)

VII. Malignant Hyperthermia

VIII. The Calpain System

IX. Summary

Water-holding Capacity

Water-holding capacity is a major quality attribute of fresh pork and can negatively impact the pork industry. Water-holding capacity is defined as the ability of meat to retain its water despite the application of force (van Laack, 1999). A decrease in water-holding capacity can result in lost product weight and thus lost financial value; further fabrication of meat into steaks and chops can result in a drip loss of 2-10% (Offer and Knight, 1988b). Additionally, it impacts the appearance of the fresh product, consumer perception at the time of purchase, and the sensory properties of the cooked product.

Lean meat contains approximately 75% water at the time of slaughter (Offer and Knight, 1998a). As 80% of the muscle fiber is composed of myofibrils, it can be assumed that the largest compartment for water in the muscle fiber is the myofibrils (Offer and Knight, 1988a). An important interaction exists between proteins and water molecules as a result of the electron distribution of the water molecules and the charged reactive groups of the proteins. The water molecule is a strong dipole with positive hydrogen and negative oxygen ends that allow the molecule to form bonds with hydrophilic groups on the proteins within the myofibril as well as with other water molecules.

Based on the water-protein and water-water interactions, cellular water can be divided into constitutional, interfacial, and bulk states (Hamm, 1986; van Laack, 1999). The smallest portion (<0.1%) of the total tissue water is considered constitutional water, which is bound very tightly to the protein through hydrogen bonding and can actually be found within the protein (van Laack, 1999). The constitutional water attracts the interfacial water at the surface of the proteins and is thus immobilized due to the strong attraction of the interfacial water to the protein-bound water (van Laack, 1999). Another portion of water (5-15%) is

held in the interfacial state (Hamm, 1986). Interfacial water is located on the surface of the protein and is held by water-water or water-protein interactions. The state that undergoes weak water-to-water interactions is bulk or "free" water (van Laack, 1999). Tissue water in the bulk or "free" state is held only by weak capillary forces. This state of water is the most abundant form found within the myofibril (Offer and Knight, 1988a).

Water can also be found in the extracellular spaces of the tissue. Swelling of the cell results in decreased extracellular space and thus an increased movement of the extracellular water into the cell causing an increase in intracellular water (Hamm, 1986). Shrinking of the cell occurs when there is increased movement of the intracellular water to the extracellular spaces (Hamm, 1986; Offer and Knight, 1988b). Shrinking and swelling can be influenced by rigor mortis development, pH, an addition or removal of monovalent or divalent cations, and aging (Hamm, 1986). It is lateral shrinking and the consequential movement of intracellular water to extracellular spaces that results in a reduction of water-holding capacity and an increased drip loss. Clearly, the balance between intra- and extracellular water is dependant on cellular shrinking and swelling. This balance is fragile and can be easily affected by genetics, pre-slaughter stress, carcass cooling, rate and extent of pH decline, and proteolysis. It is important to understand the factors that affect water-holding capacity as information on the mechanisms involved will provide better means of controlling and predicting this quality trait.

Water-holding capacity can be greatly affected by biochemical processes. The decrease in pH immediately postmortem is a very important factor. Rapid postmortem glycolysis results in an accumulation of lactate and hydrogen ions. This would thus result in a faster rate of pH decline while the muscle temperature is still high, which can reduce water-

holding capacity. Lonergan, Huff-Lonergan, Rowe, Kuhlers and Jungst (2001) showed that pigs that were selected for lean growth efficiency had a significantly faster rate of pH decline from the control line. Additionally, pigs that displayed a rapid pH decline also had a greater amount of drip loss from the longissimus dorsi after 1, 3 and 4 days of storage; the semitendinosis at 2, 3 and 4 days of storage; and the semimembranosis at 1, 2, 3 and four days of storage (Lonergan et al., 2001).

Rapid pH decline in pork muscles, combined with a high carcass temperature within the first hour postmortem causes the proteins in the muscles to denature (Honikel and Kim, 1986; Offer and Knight, 1988b; Offer, 1991) which contributes to pale color (Honikel and Kim, 1986) and poor water-holding capacity (Offer and Knight; 1988b). Denaturation of contractile proteins causes the distance between actin and myosin to shrink due to the length of the myosin heads decreasing from 19 nm to 17 nm (van Laack, 1999). Altering the cross bridges formed by actin and myosin causes the filament spacing to decrease and more intracellular water would be expelled into the extracellular spaces.

Low water-holding capacity is also associated with a low ultimate pH in the meat. The normal ultimate pH of the longissimus dorsi in pork muscle is 5.5-5.6 (Winger and Pope, 1980-81). As the ultimate pH approaches the isoelectric point, or the point at which there is no net charge on the protein (~pH 5.0-5.1), the ability of the protein to bind the dipolar water molecule is reduced (Wismer-Pederson, 1978). Additionally, the proteins in the tissue would have less charge for repulsion and the space between the thick and thin filaments would decrease.

Degradation of specific proteins would also significantly affect water holding capacity. Kristensen and Purslow (2001) proposed that degradation of the cytoskeleton of

the muscle fiber during aging might increase water-holding capacity by removing inter-myofibrillar and costameric connections and open up the structure. Removal of the connections would reduce or remove the rigor-induced lateral shrinkage of myofibrils and prevent the shrinkage of the whole muscle fiber, resulting in a movement of water from intracellular to extracellular compartments which could result in improved water-holding capacity during later postmortem times (Kristensen and Purslow, 2001). Rowe, Lonergan, Rothschild, and Huff-Lonergan (2001b) supported this hypothesis by showing that degradation of the intermediate filament protein desmin correlated with reduced drip loss at two and five days postmortem in pork samples. A study reported by Dodge (2002) also provided significant evidence as, in this study; the pork muscles with most proteolysis of desmin had the least amount of drip.

The onset of rigor mortis also affects water-holding capacity. At rigor, lateral shrinkage of myofibrils causes lateral contraction when myosin heads bind to actin to form actomyosin (Offer et al., 1989). The filament spacing is reduced and drip channels are generated along the perimysium (Offer and Knight, 1988a, b; Offer and Cousins, 1992).

In order to understand where cellular water is held in the muscle, the structure of skeletal muscle will be discussed in the following section of this literature review.

Overview of the Structure of Skeletal Muscle

Skeletal muscle is the most abundant kind of muscle tissue found in meat animals and makes up approximately 40% of the total body weight (Goll, Robson and Stromer, 1984). Skeletal muscle is a voluntary, striated muscle and both its macro- and micro-structure are complex.

A cross section of the entire muscle shows it is surrounded by a sheet of connective tissue called the epimysium, a relatively thick and tough structure that is the site of adipose tissue deposition (Goll et al., 1984). Additional sheets of connective tissue, the perimysium, intermittently pass into the muscle and divide it into bundles of muscle fibers or fasciculi. The size of the muscle bundles is dependent on the use of the muscle (Goll et al., 1984). Blood vessels and nerves can be found contained by the perimysium. The endomysium is the next set of connective tissue sheets and it extends from the perimysium to surround each individual muscle fiber, or myofiber (Goll et al., 1984). The endomysium is very delicate and runs parallel to the outer cell membrane called the sarcolemma. The sarcolemma is elastic, which allows the muscle fibers to change shape during contraction, relaxation and stretching (Aberle, Forrest, Gerrard and Mills, 2001).

The functional units of the skeletal muscle are the muscle fibers, which are long, multinucleated, unbranched, threadlike cells that taper at each end. They may range in 1-340 mm in length but, in general, do not extend the length of the entire muscle. The myofibers can range in diameter at their centers from 10 to 100+ μm (Huxley, 1963). The diameter can vary within a species and/or within the muscle itself. The myofibers contain myofibrils that are surrounded by the sarcoplasm (cytoplasm) of the cell (Cassens, 1987).

The sarcolemma, nuclei, sarcoplasm, mitochondria and myofibrils are all a part of the muscle fiber (Cassens, 1987). The sarcolemma (cell membrane) surrounds the fiber and is composed of two distinguishable parts, the plasmalemma and the basement membrane (Goll et al., 1984). The sarcolemma is chemically composed of proteins and lipids and is relatively elastic, which contributes to its ability to become greatly distorted during contraction, relaxation and stretching (Aberle et al., 2001). The sarcolemma has invaginations that occur

along the length and around its circumference that form a network of tubules called transverse tubules, or T-tubules (Goll et al., 1984). The T-tubules connect to the terminal cisternae of the sarcoplasmic reticulum (SR) and are open to extracellular space. The structures are responsible for conducting a neural signal from the sarcolemma to the interior of the muscle cell. The T-tubule system aids in the sarcolemma's role in propagating excitation-contraction coupling (Goll et al., 1984). The sarcoplasmic reticulum is also considered a tubule system in skeletal muscle. The transverse tubules are often discussed in combination with the sarcoplasmic reticulum, but are important to realize the structures are separate. The sarcoplasmic reticulum is a system of membranous tubules and cisternae that extends from one T-tubule to another and surrounds individual myofibrils. This system is able to accumulate Ca^{2+} against a concentration gradient as well as split ATP in order to release energy. Splitting one molecule of ATP by the sarcoplasmic reticulum is coupled to the transport of two molecules of Ca^{2+} to the lumen of the sarcoplasmic reticular tubule (Goll et al., 1984). By later releasing the accumulated calcium when a signal is produced by the passage of an action potential and causing an increase in free intracellular Ca^{2+}, the sarcoplasmic reticulum plays an important role in triggering muscle contraction. The myofibers are multinucleated, containing 100 to 200 nuclei per cell. Nuclei in skeletal muscle are located at the periphery of the fiber, just below the sarcolemma. Sarcoplasm is the cytoplasm found in muscle cells and differs from nonmuscle cytoplasm as it contains an oxygen-storage protein called myoglobin and also has slightly different glycolytic enzyme content (Goll et al., 1984). It is important to note that the sarcoplasm is 75-80% water and also contains lipid droplets, glycogen granules, ribosomes, numerous proteins, nonprotein nitrogenous compounds and a number of inorganic constituents (Aberle et al., 2001). There

are many mitochondrial structures present at the periphery of muscle cells and they use most of the oxygen stored in the cell (Goll et al., 1984).

The myofibril is unique to skeletal muscle and is responsible for the contractile properties of muscle cells. They are surrounded by sarcoplasm and run the entire length of the muscle fiber. These structures pack the myofibers; 80-87% of the interior of muscle cells consists of myofibrils (Goll et al., 1984). When studied under a polarizing microscope that detects areas of different density, myofibrils have a striated pattern consisting of light and dark bands. The dark bands are birefrigent or anisotropic (double refractive) and are termed to be the A bands while the light bands are weakly birefrigent and isotropic (singly refractive) and are called the I bands (Aberle et al., 2001). When stained and viewed with an electron or phase microscope, the I band is shown to be bisected by a dark line called the Z disk (Z-line). A light zone called the H zone can be seen in the middle of the A band, which is bisected by the M line (Goll et al., 1984; Cassens, 1987). Additionally, when the muscle is relaxed, the I band can be shown to have a N_2-line (Goll et al., 1984). The section between two Z-lines is called a sarcomere and includes both an A band and half of the two I bands located on either side of the A band. The sarcomere is the site where muscle contraction and relaxation occurs.

Thick and thin filaments are also organized within the myofibrils. They differ from one another not only in diameter as their names suggest, but also in chemical composition, properties, and location within the sarcomere (Aberle et al., 2001). The thick filaments are 14-16 nm in diameter and 1.5 μm in length while the thin filaments are 6-8 nm in diameter and 1 μm in length (Goll et al., 1984). The thick filaments make up the A band of the sarcomere. The myosin found in the myofibril is located in the thick filament (Cassens,

1987). The thick filaments are also made up of paramyosin, C-proteins and H-proteins (Goll et al., 1984). The thin filaments constitute the I band of the sarcomere and extend beyond the I band into the A band where they overlap the thick filaments (Goll et al., 1984). The thin filaments are called the actin filaments as they are composed predominately of actin, but also contain tropomyosin, troponin, and tropomodulin (Aberle et al., 2001).

Muscle Contraction/Relaxation and Calcium Signaling

The primary role of muscle is to provide a means of movement through carefully coordinated contraction/relaxation cycles modulated by fluctuations in cytosolic calcium levels. Contraction of striated muscle results from a rise in cytoplasmic calcium concentration. Most of this calcium moves back and forth across the SR membrane in cycles of contraction and relaxation. Ca^{2+} is the primary regulator of the contraction/relaxation cycle. During contraction, Ca^{2+} is released from the sarcoplasmic reticulum and Ca^{2+} levels within the sarcoplasm reach approximately 10^{-5} M. During relaxation, the concentration is lowered to about 10^{-7} (Bowker, Grant, Forrest and Gerrard, 2000).

Muscle contraction is initiated and sustained by a nerve impulse that is propagated along a motor neuron to the neuromuscular junction, or the motor end-plate. (Goll et al., 1984). This nerve impulse causes acetylcholine to be released from presynaptic vesicles in the axon terminus into the synaptic cleft. The released acetylcholine binds to postsynaptic nicotinic acetylcholine receptors which cause Na^{+} to depolarize the sarcolemma at synaptic sites where the acetylcholine receptors are located (Missiaen et al., 2000). Depolarization is propagated through the sarcolemmal membrane to the transverse tubule (T-tubules) and to the voltage sensitive dihydropyridine (DHP) receptor located on the T-tubule. DHP

receptors function by interacting with the Ca^{2+}-releasing channel, the ryanodine receptor (RyR). The RyR is located on the terminal cisternae of the sarcoplasmic reticulum, which are closely apposed to the T-tubules. The site where the terminal cisternae of the sarcoplasmic reticulum attach to the invaginated T-tubules system of the plasma membrane via the RyR is called the junctional triad. The signal transduction process that occurs in the triad is known as excitation-contraction coupling. The DHP receptors open and allow small amounts of calcium into the cell but does not raise intracellular calcium levels sufficient for contraction, rather it functions to open the RyR which releases the calcium required to stimulate muscle contraction (Protasi, 2002). Upon stimulation, the RyR elevates cytosolic Ca^{2+} locally to approximately 100-fold higher levels (Berchtold, Brinkmeier and Müntener, 2000).

In order to continue the contraction process, free Ca^{2+} released into the sarcoplasm from the ryanodine receptor binds in a very rapid reaction to the troponin complex in the thin filament and a conformational shift occurs. The troponin complex consists of troponin-I, troponin-T, and troponin-C. In the absence of Ca^{2+}, troponin-C is loosely bound to troponin-I and troponin T; troponin-I is firmly bound to actin and loosely bound to troponin-T; and troponin-T is strongly bound to tropomyosin (Goll et al., 1984). Thus, the tropomyosin blocks the myosin-binding site of actin in the absence of Ca^{2+}. However, in the presence of Ca^{2+}, troponin-C becomes strongly bound to troponin-I and troponin-T, and troponin-I loses its affinity for actin while retaining the loose linkage to troponin-T (Goll et al., 1984). As a result, the tropomyosin strand shifts and exposes the myosin-binding site, which allows myosin cross bridges to interact with actin. The actin filaments and the Z-lines are pulled toward the center of the sarcomere (Goll et al., 1984) and contraction occurs. Contraction

decreases the length of the I-band and H-zone, but does not change the distance from Z-disks to the nearby H-zone or the length of the A-band (Garrett and Grisham, 1999).

For relaxation, ADP and inorganic phosphate, the hydrolysis products of ATP bound to myosin, must be released from myosin. After ADP has been released, a new molecule of ATP must bind to the myosin head to allow it to relax; and the myosin cross bridge must dissociate from actin (Goll et al., 1984). This series of events occurs until the sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) located on the sarcoplasmic reticulum membrane reaccumulates the free Ca^{2+} into the lumen of the sarcoplasmic reticulum; thereby removing the ability of Ca^{2+} to induce contraction (Missiaen et al., 2000). The SERCA pump is triggered by the increased cytosolic calcium concentration. In the absence of Ca^{2+}, troponin-C no longer binds Ca^{2+}, troponin-I interacts directly with actin (Garrett and Grisham, 1999); and tropomyosin moves out of the actin helix groove to cover the myosin binding sites on actin (Goll et al., 1984).

Muscle Metabolism and Rigor Mortis

The key metabolic pathway in the conversion of muscle to meat is postmortem glycolysis. In living tissue, metabolism is mostly aerobic and the production of ATP is derived from the TCA cycle. However, in postmortem tissue, the muscle attempts to maintain homeostasis by preserving cellular ATP concentrations, but due to failure of the circulatory system, muscle lacks the oxygen required for oxidative metabolism. Additionally, ATP combined with Mg^{+} is required for muscle to be maintained in an relaxed state. During early postmortem periods, stores of creatine phosphate are used to rephosphorylate ADP to ATP; however, these stores quickly become insufficient

phosphorylation to help maintain relaxation of the muscle. Thus, metabolism is converted to the anaerobic system of glycolysis and ADP is phosphorylated by mobilization of glycogen stores in order to replenish ATP. Glycolysis is the pathway for providing energy from the stepwise degradation of glucose (Garrett and Grisham, 1999) and produces short term energy when oxygen is limited to the organism. The rate of glycolysis is strongly affected by rate of postmortem cooling, rigor onset and temperature decline (Marsh, 1954, as cited by O'Halloran, Troy, Buckley and Reville, 1997). Due to its effect on temperature and pH, the rate of glycolysis can also influence proteolytic enzyme activity (Koohmaraie, Schollmeyer and Dutson, 1986; Dransfield, 1992).

Glycolysis can be broken down into two series of reactions. The first phase of glycolysis consumes two molecules of ATP and involves a series of five reactions in which glucose if broken down into two molecules of glyceraldehyde-3-phosphate and results in the consumption of two molecules of ATP (Garrett and Grisham, 1999). The second phase converts the two molecules to glyceraldehyde-3-phosphate into two molecules of pyruvate and results in a net gain of four ATP molecules. Thus, glycolysis has an overall net gain of two ATP molecules. ATP plays large role in muscle contraction and metabolism as it provides energy for dissociating actin-myosin cross-bridges, for muscle contraction, and for maintaining the SERCA pump (Goll et al., 1984). If ATP is not available in the muscle fiber, irreversible cross bridges form between myosin heads and actin and a state called rigor mortis occurs; contraction depends on the rapid ability of the muscle to produce ATP. Rigor development is also accompanied by the muscle losing its ability to reaccumulate Ca^{2+} and maintain the Ca^{2+} gradients; thus, an increase in sarcoplasmic calcium concentration occurs (Mickelson and Louis, 1993).

Glycolysis accounts for the reduction of pH in postmortem muscle. Exsanguination results in a lack of oxygen between the circulatory system and muscle tissue due to a loss in the function of the circulatory system. As oxygen is depleted, a shift from aerobic to anaerobic metabolism occurs as the body is still trying to produce ATP and maintain function which results in less efficient production of ATP while it is still being consumed in the muscle. Under anaerobic conditions, two molecules of pyruvate produced by glycolysis are reduced to lactate. As oxidation is not possible, pyruvate is then reduced by lactate dehydrogenase to produce lactic acid. Lactic acid accumulates as the circulatory system is not able to remove the lactic acid to the liver in order to convert it back to glucose (Goll et al., 1984), and muscle pH declines from 7.4 at exsanguination to approximately 5.5-5.8. The rate of pH decline is related to the temperature of the muscle. A higher temperature will cause a faster rate of pH decline while a lower muscle temperature will cause a slower rate of pH decline. The rate of pH decline can be affected by genetics, pre-slaughter stress, diet, and postmortem processing.

The Sarcoplasmic Reticulum Ca^{2+}-ATPase Pump

The sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) pump is a part of a large family of cation transporters, P-type ATPases, which are thus named because they contain an aspartate residue that is phosphorylated by ATP in the presence of the appropriate cation (Møller, Juul and le Maire, 1996). This 110 kDa transmembrane protein is expressed at high levels in cardiac, skeletal, and smooth muscle cells (MacLennan, 1970) and makes up 90% of the membrane protein. The sarcoplasmic reticulum Ca^{2+}ATPase pump plays a key role in calcium signaling as it is responsible for recharging the SR/ER stores and for bringing

calcium levels down to baseline levels thus permitting relaxation following Ca^{2+} release. This calcium pump will function as long as ATP is present. The proper function of the sarcoplasmic reticulum Ca^{2+}-ATPase pump is especially crucial during the early postmortem period when the calcium levels in the sarcoplasm must be maintained at a low concentration in order to prevent an increased rate of glycolysis and a faster pH decline (Mickelson and Louis, 1993). Failure to accomplish this mechanism in postmortem pork could result in poor water-holding capacity.

Structure of the SERCA pump. The structure of this calcium regulating pump consists of a single polypeptide chain that is folded into four major domains: a transmembrane domain (M) comprised of an arrangement of ten transmembrane helices (M1-M10) and three cytosolic domains (A-actuator, N-nucleotide binding, and P-phosphorylation), with most of the structure residing in the sarcoplasm (MacLennan, Brandl, Korczak and Green, 1985; Mickelson and Louis, 1993). Studies have implicated that acidic and hydrophobic residues in the middle of transmembrane M4-M6 and M8 are involved in calcium binding (Andersen, 1995; Møller et al., 1996; MacLennan, Rice, Odermatt and Green, 1997). Together, the three cytosolic domains form a compact headpiece structure in the E2 state held together by a crystallization agent (Stokes and Wagenknecht, 2000). Each domain has a different function. The N domain is believed to bind ATP while the P domain accepts a phosphoryl group on its aspartate 351 residue. The A domain is located between the transmembrane helices M2 and M3 and includes the N-terminus. The A domain's function is currently unclear, but it is considered to be the actuator for the N domain and it has been suggested to play a role in transferring energy from the P and N domains through to the calcium binding sites (Møller et al., 1996). Two of the cytosolic domains, N and P, are connected to the M domain.

Mode of action of the SERCA pump. The catalytic cycle of the SERCA by which calcium is taken up from the cytoplasm and pumped against a concentration gradient into the sarcoplasmic reticulum involves two major conformational states, E1 and E2. The E1 conformation has a high affinity for calcium from the cytoplasm and uses ATP readily to phosphorylate the catalytic aspartate residue. The calcium binding sites in the E1 conformation face the luminal side of the sarcoplasmic reticulum. The E2 conformation has a low affinity for calcium ions and the nucleotide site is unreactive to ATP. The calcium binding sites on this conformation faces the cytoplasmic side of the cell. By altering the affinity of the Ca^{2+}-binding sites from high (E1) to low (E2), active transport of Ca^{2+}-ATPase can be achieved (Toyoshima, Nakasako, Nomura and Ogawa, 2000).

First, two Ca^{2+} ions are removed from the cytoplasm and bound to the E1 state and ATP is bound to the N domain. If the Ca^{2+} concentration is high in the sarcoplasm and bound to the E1 phase, ATP can then be cleaved and a phosphate group is transferred to the Asp-351 on the P domain to result in the E1-P conformation. The ion binding sites are inverted to form the E2-P conformation, allowing the calcium ions to only dissociate into the sarcoplasmic reticulum and not the sarcoplasm. The calcium ions are subsequently released to the luminal side of the membrane. Upon the release of the Ca^{2+} ions, the phosphoryl group is hydrolyzed and released from the P domain which changes the pump to the low affinity E2 conformation. As the E2 confirmation is unstable without the phosphoryl group bound, the pump returns to E1 form, which thus allows additional Ca^{2+} to be removed from the sarcoplasm.

Isoforms of the SERCA pumps. The calcium pumps are encoded by three genes that, after differential splicing, give rise to at least seven different isoforms: SERCA1a/b,

SERCA2a/b, and SERCA3a/b/c (Møller et al., 1996; Wuytack, Dode, BabaAissa and Raeymaekers, 1995). SERCA1a is predominately found in adult fast-twitch skeletal muscle while SERCA1b exists in neonatal muscle and accounts for ~70% of the fast-twitch Ca^{2+}-ATPase prior to birth (Brandl, Green, Korckzak and MacLennan, 1986; Brandl, deLeon, Martin and MacLennan, 1987). The isoform SERCA2a is expressed in cardiac muscle and its expression level increases from neonatal to adult stages (MacLennan, et al., 1985). SERCA 2a is also found in slow-twitch skeletal muscle and fetal fast-twitch muscle as well as smooth muscle (Brandl et al., 1987). It is important to note that in adult fast-twitch muscle, SERCA2a is replaced by the isoform SERCA1a (Brandl et al., 1987). SERCA2b is found in most all nucleated cell types with the most expression of this isoform is in smooth muscle (Lytton, Zarain-Herzberg, Periasamy and MacLennan, 1989). SERCA3a and 3b are found in specialized cells such as mast cells, platelets, lymphocytes, endothelial and epithelial cells (Wuytack et al., 1995).

The Ca^{2+}-ATPase isoforms are differentially expressed and have unique functional properties. SERCA1a and 2a have similar apparent affinities for calcium and similar protein turnover numbers, but in comparison, SERCA2b has a lower protein turnover number and SERCA3 has a lower affinity for calcium (Lytton, Westlin, Burke, Shull and MacLennan, 1992). Additionally, SERCA2b has a two-fold higher calcium binding affinity than 2a, although its protein turnover number is two-fold lower (Verboomen, Wuytack, Desmedt, Himpens and Casteels, 1992). A possible reason behind the differences is that fast-twitch skeletal muscle has a requirement for a faster turnover, thus it would be necessary for the isoform (SERCA1a) predominately present in the tissue to also have a faster turnover rate. SERCA1a is expressed at three- to five-fold higher levels in adult fast-twitch skeletal muscle

than SERCA2a levels in slow-twitch or cardiac muscle (Wu and Lytton, 1993). This difference may account for the different Ca^{2+} uptake capacities in fast- and slow-twitch skeletal muscle (Lytton et al., 1992).

Regulation of the SERCA pump. Modulation of calcium pump activity can have profound effects on calcium signaling, thus it is important to note the mechanisms that regulate sarcoplasmic reticulum Ca^{2+}-ATPase pumps. Phospholamban, an intrinsic membrane protein that contains 52 amino acid residues, is able to inhibit SERCA1 and SERCA2a/b. The N-terminus is hydrophilic and located in the cytoplasm while the C-terminus in hydrophobic in nature and is believed to be positioned near the center of the transmembrane section of SERCA2 (Hutter et al., 2002). Phospholamban has the potential to up-regulate SERCA1 and SERCA2a/b. However, only the isoforms SERCA2a/b can be regulated in this manner as phospholamban is not found in fast-twitch skeletal muscle (Fujii, Lytton, Tada and MacLennan, 1988). This protein can only weakly regulate SERCA3 (Toyofuku, Kurydlowski, Tada and MacLennan, 1993). When phospholamban is phosphorylated by protein kinase A and/or calmodulin kinase, its inhibitory affects are removed (Kimura, Kurzydlowski, Tada, and MacLennan, 1996; Levine et al., 1999).

A transmembrane protein similar in sequence to phospholamban, sarcolipin, appears to regulate SERCA1. Most abundant in fast-twitch skeletal muscle, this 31 amino acid peptide is co-expressed with SERCA1 which reduces the affinity of the isoform to bind Ca^{2+}, but increases the V_{max} for calcium transport (Odermatt et al., 1998). Unlike phospholamban, phosphorylation does not seem to alter sarcolipin's inhibitory affect. Rather, it is believed that altering the expression of sarcolipin by binding to phospholamban in the sarcoplasmic

reticulum is the mode by which the protein exerts control over SERCA1 (Odermatt et al., 1998).

There is also evidence to suggest that SERCA2a and 2b may be up-regulated by phosphorylation of Ser 38 (Xu, Hawkins, and Narayanan, 1993). The isoforms of SERCA1 and SERCA3 are not regulated by this mechanism as the Ser 38 residue is not conserved in the isoforms (Dode et al., 1996). A range of studies have shown that phosphorylation of this residue resulted in up to a two-fold increase in the calcium transport rate (Xu et al., 1993; Allen and Katz, 1996; Grover, Xu, Samson and Narayanan, 1996; Osada, Netticadan, Tamura and Dhalla, 1998).

Abnormalities of the SERCA pump. Loss of SERCA1a Ca^{2+} pump function is one cause of exercise-induced impairment of the relaxation of skeletal muscle, which is the main defect of Brody disease, a rare inherited disorder of skeletal muscle function (Odermatt et al., 1996; MacLennan et al., 1998). Ca^{2+} uptake and Ca^{2+} ATPase activities are reduced in the sarcoplasmic reticulum, leading researchers to believe that Brody disease results from defects in SERCA1. More studies are needed to clarify the exact origin and mechanisms behind this disease.

The Ryanodine Receptor

Calcium is a fundamental second messenger that regulates many processes in the cell. Although cells are typically surrounded by solutions containing relatively high Ca^{2+} concentrations, the cytoplasm of most cells contains much lower resting Ca^{2+} concentrations. Thus, Ca^{2+} entry across the surface membrane can substantially elevate Ca^{2+} levels in the cytosol, providing the Ca^{2+} trigger signals for a large number of physiological processes.

Entry across the surface membrane is not the only way Ca^{2+} signals can be triggered. Other pathways involving specialized Ca^{2+} storage and release organelles are involved.

Isoforms of the RyR. The sarcoplasmic reticulum, the primary intracellular Ca^{2+} storage/release organelle in striated muscle cells, contains a key protein involved in excitation-contraction coupling. The ryanodine receptors (RyR) act as calcium release channels and are characterized by their large size (565 kDa per subunit). Calcium is released in response to surface membrane depolarization. The ryanodine receptor was identified and isolated through its high affinity to the toxic plant alkaloid, ryanodine, which modulates channel opening (Lai, Erickson, Rousseau, Liu and Meissner, 1988), hence the origin of the name of the protein.

There are three known isoforms: skeletal RyR1, cardiac RyR2, and brain RyR3 (Fill and Copello, 2002). The primary structures of the isoforms share approximately 70% sequence identity of amino acid composition (Hakamata, Nakai, Takeshima and Imoto, 1992; Takeshima et al., 1989). The predominant RyR isoform in skeletal muscle is RyR1, although RyR3 is also found in mammalian striated muscles at relatively low levels (Froemming, Murray, Harmon, Pette and Ohlendieck, 2000; Sutko et al., 1991). The physiological role of RyR3 in skeletal muscle is unknown. However, in a study conducted by Takeshima and others (1998), mice missing the RyR1 and RyR2 gene products died during embryonic development, but mice missing the RyR3 gene product survived and had normal striated muscle.

Structure of the RyR. The RyRs are large oligomeric structures that are formed by the association of four RyR subunits. At 565 kDa per polypeptide subunit, the RyRs are the largest ion channels currently known. The RyR also contains four tightly associated copies of

a much smaller 12 kDa protein, FK-506 binding protein, an immunophilin, whose role in receptor function remains to be clarified (Timmerman et al., 1993). Skeletal RyR are located principally at specialized regions, or triad junctions, where the sarcoplasmic reticulum and transverse tubules are apposed (Franzini-Armstrong and Jorgensen, 1994). The secondary structure suggests that transmembrane sequences are located in the C-terminal end of the RyR while the rest of the protein is cytoplasmic and bridges the gap between the T-tubule and the sarcoplasmic reticulum (Best, Kwok and Xu, 1991). The cytoplasmic portion of the RyR is the foot structure that bridges the small 10 nm gap between the sarcoplasmic reticulum and the T-tubules (Froemming et al., 2000).

The 3-D structure of the RyR shows a fourfold symmetry, presumably reflecting its formation by four RyR protein monomers (Wagenknecht et al., 1989). The RyR has two distinct domains. One domain is a large cytoplasmic assembly, consisting of a loosely packed assembly of domains, while the other domain is a small transmembrane assembly that protrudes from the center of the cytoplasmic assembly (Wagenknecht et al., 1989). This transmembrane assembly appears to have a central hole that can be occluded by a pluglike mass (Wagenknecht et al., 1989). The hole and plug may correspond to the Ca^{2+}-selective pore and its gate. Cryoelectron microscopy studies have revealed the sites where certain peptides such as calmodulin and FK binding protein interact with the RyR (Wagenknecht and Radermacher, 1997). Also, a specific domain at amino acids 4425-4621 may be implicated in the Ca^{2+}-dependent regulation of the RyR channel (Benacquista et al., 2000).

Interaction with the DHP receptor. In skeletal muscle, the passage of Ca^{2+} through the RyR is believed to involve a protein-protein interaction with the dihydropyridine (DHP) receptor found on the T-tubule (Campbell et al., 1987; Proenza et al., 2002); the signal for

Ca^{2+} release is thought to be passed on to the RyR by the DHP. The DHP tetrad receptor is similar to the voltage dependent sodium channels as each of the four repeated segments has five hydrophobic segments and one positively charged segment in its alpha one subunit (Tanebe et al., 1987). It is the positive charge in this segment that may act as a voltage sensor. The skeletal DHP are arranged in clusters of tetrads located on the corners of small squares. The squares are organized into distinct arrays that correspond with the array of the RyR which allow the DHP to directly affect the RyR during excitation-contraction coupling. In fast-twitch muscle, the DHP receptors are aligned so every other RyR1 channel is associated with a DHP tetrad (Protasi, 2002). In cardiac muscle, DHP receptors induce nearby RyR to open by calcium-induced calcium release (CICR). However, in skeletal muscle, there is evidence that DHP receptors physically interact with RyR at the triad junction (Brandt, Caswell, Wen and Talvenheimo, 1990).

Regulation of the RyR. Analysis of the RyR's primary amino acid sequence has revealed several consensus ligand binding and phosphorylation motifs. The RyR is thought to be regulated and modulated by numerous ligands such as T-tubules, Ca^{2+}, Mg^{2+}, calmodulin, FK-506 binding protein and adenine nucleotides. Ligands such as micromolar Ca^{2+}, caffeine and millimolar ATP stimulates Ca^{2+} release, ryanodine binding, and channel opening. Ligands such as ruthenium red, calmodulin and millimolar Mg^{2+} or Ca^{2+} close the channel, inhibit Ca^{2+} release, and inhibit ryanodine binding. Various cellular processes such as phosphorylation and oxidation also modulate the Ca^{2+}-release channel. Phosphorylation has been show to increase RyR activity (Coronado, Morrissette, Sukhareva and Vaughan, 1994), whereas oxidation of the RyR has been suggested to allow regulatory molecules to bind to RyR and subsequently decrease activity (Zhang et al., 1999).

Calmodulin, an ubiquitous intracellular Ca^{2+} receptor that contains EF-hand structures that bind calcium in a specific way, is a macromolecular modulator of RyR. Calmodulin binds to the RyR and negatively affects its function (Seiler, Wegener, Whang, Hathaway and Jones, 1984). There is one calmodulin molecule to four sites per RyR subunit (Mackrill, 1999). Wagenknecht, Berkowitz, Grassucci, Timmerman and Fleischer (1994) found that calmodulin, which regulates the RyR by direct interaction in vitro, binds to a site on each subunit. At submicromolar Ca^{2+} concentrations, calmodulin activates the RyR by increasing the open probability in a dose-dependent manner (Buratti, Prestipino, Menegazzi, Treves and Zorzato, 1995). At high free Ca^{2+} concentrations, calmodulin inhibits RyR activity (Buratti et al., 1995).

Ca^{2+} and pH-dependent conformational changes in calsequestrin may modulate RyR activity. Calsequestrin is the main binding protein inside the SR and has a large number of amino acid sites that permit it to bind 40-50 Ca^{2+} molecules per one calsequestrin molecule; calsequestrin clearly is a high capacity, low affinity Ca^{2+} binding protein (Wang, Villalobo, and Roufogalis, 1989). The rate of Ca^{2+} release from skeletal muscle SR is dependent on the Ca^{2+} loading of these membranes, which is largely dependent on Ca^{2+} binding to calsequestrin (Mackrill, 1999). Jones and others (1998) found that transgenic mice exhibiting tenfold over-expression of cardiac calsequestrin developed severe cardiac hypertrophy and had a twofold increase in heart mass and size. Mice with overexpressed calsequestrin had repressed RyR activity (Jones et al., 1998), suggesting an impairment of the Ca^{2+} release mechanism.

Malignant Hyperthermia

Because Ca^{2+} is such an important signaling molecule, mutations causing functional changes in intracellular Ca^{2+} homeostasis can trigger serious and possibly fatal consequences. An abnormality of the ryanodine receptor, malignant hyperthermia (MH), is an example of such consequences. The defect in MH instigates hypersensitive gating of the Ca^{2+}-release channel (RyR) in the skeletal muscle SR; channel opening is facilitated while closing is inhibited (Fujii et al., 1991).

Certain strains of pigs have a condition termed Porcine Stress Syndrome (PSS) or Malignant Hyperthermia (MH). PSS is caused by a mutation in the ryanodine receptor where the amino acid sequence is altered from an arginine at position 615 in the Malignant Hyperthermia Normal (MHN) animals to a cysteine in Malignant Hyperthermia Susceptible (MHS) animals (Fujii et al., 1991). The mutated Ca^{2+}-release channel is abnormally permeable to Ca^{2+}, even in basal conditions. Due to an abnormal Ca^{2+} release, the resulting continuous small muscle contractions lead to muscle hypertrophy, and the increased ATP consumption during these spontaneous contractions limits the deposition of fat. Thus, MHS pigs are often associated with leanness and muscle hypertrophy, which adds economic incentive based on lean carcass weight. As the industry has encouraged production of growth and muscle, selection is inadvertently being made for the MH gene. Unfortunately, MHS pigs can be negatively triggered by physical or physiological stress which can cause death and pale, soft, and exudative (PSE) meat.

In steady-state conditions, the intracellular Ca^{2+} stores must reaccumulate the same amount of Ca^{2+} as they release. However, the hyperactivity of the mutated RyR channels during stress floods the cells with Ca^{2+} and overpowers the SERCA pumps that would

normally function to lower Ca^{2+} concentrations and thus cease continued contraction. The continuous activation of the SERCA pumps and actomyosin ATPases leads to the continuous hydrolysis of ATP accompanied by substantial heat production and hyperthermia. The elevated Ca^{2+} concentration also stimulates an enhanced glycolysis and an accelerated anaerobic metabolism that could quickly deplete ATP, glucose and oxygen; produce excess CO_2, lactic acid and heat; and upset cellular and extracellular ion balances (MacLennan and Phillips, 1992). An excess production of lactate leads to metabolic acidosis, and carbon dioxide overproduction leads to respiratory acidosis.

Malignant hyperthermia susceptible (MHS) and malignant hyperthermia resistant pigs have been studied to evaluate the effects that stress imparts on the cellular events that regulate Ca^{2+} release and uptake. Küchenmeister and others (1999a) used MHS and MHN genotypes to provide information on the development of cell injury during the postmortem time period and its effects on meat quality. They found that MHS animals had a lower pH at 45 min postmortem. Additionally, MHS animals reached the ultimate pH within 4 h postmortem compared to 22 h in the MHN muscle. However, no significant differences between genotypes were observed between the ultimate pH measurements. A higher drip loss and higher Minolta L* values were found in MHS longissimus compared to that of MHN muscles. It was also observed that the permeability of the sarcoplasmic reticulum membranes to Ca^{2+} was increased and greater Ca^{2+}-ATPase activity was displayed at 45 min and 4 h postmortem in MHS animals. This would suggest that more Ca^{2+} is being released into the cell in the MHS animals, thus the cell is attempting to maintain the proper level by increasing the activity of the Ca^{2+}-ATPase in order to return Ca^{2+} back into the sarcoplasmic reticulum. Increased extracellular space occurred at a higher rate and at an earlier time

postmortem in MHS pigs compared to MHN pigs. This may have influenced the meat quality traits by increasing the efflux of intracellular fluids into the increased extracellular space, which could then facilitate drip loss (Küchenmeister et al., 1999a).

Another study by Küchenmeister, Kuhn, Wegner, Nürnberg and Ender (1999b) showed that the ability of the sarcoplasmic reticulum to sequester Ca^{2+} decreased to approximately 60% in the first 45 min postmortem in MHS pigs compared to 5% in MHR pigs. Additionally, the authors found that the calcium release channel functions up to 22 h postmortem and is almost fully open in MHS pigs at 0 min, 45 min, 4 h, and 22 h postmortem, but is only partially open in MHN animals at the same time periods. In a study by Küchenmeister, Kuhn and Endor (2000), it was found that season affected the Ca^{2+} transport of the sarcoplasmic reticulum and subsequent meat quality. Both MHN and MHS pigs were found to have reduced Ca^{2+} uptake during the summer (June and July) season compared to the winter (November and December) season, suggesting that even in animals with normal calcium release channels, seasonal effects can impact Ca^{2+} homeostasis. The authors found that the altered Ca^{2+} regulation in MHN and MHS pigs harvested in the summer season was correlated with poorer meat quality in reference to pH at 45 min postmortem, color, and drip loss (Küchenmeister et al., 2000).

The Calpain System

The calpains are Ca^{2+} dependent cysteine proteases with an optimum pH between 7.2 and 8.2 and are ubiquitously distributed in the cytosol of all animal cells. As the calpain system appears to have a profound effect on the RyR and the SERCA pump, in addition to

having proteolytic properties that allow them to degrade proteins involved in water-holding capacity, they will be discussed in detail.

Nomenclature and structure of Calpains. The calpain system has been widely studied since m-calpain was first purified to homogeneity from porcine skeletal muscle by Dayton and others in 1976. In 1981, μ-calpain was also purified from porcine skeletal muscle by Dayton and recognized as a part of the calpain system (Goll, Thompson, Taylor, Ouali and Chou, 1999). The calpain family is a superfamily of multi-domain proteases in which the large subunits of the isoforms possess the general features of a papain-like cysteine protease and a calmodulin-like Ca^{2+} binding protein (Saido, Sorimachi and Suzuki, 1994). Hence, the name calpain was derived from the "cal" in calmodulin and the "pain" from papain.

The calpain family can be divided into three groups based on distribution. These groups are ubiquitous and tissue-specific. Currently, knowledge of the calpains applies predominantly to the ubiquitous enzymes, μ- and m- calpain, and their naturally occurring inhibitor, calpastatin (Goll et al., 1999).

Calpains and calpastatin are located exclusively intracellularly and are found on or near the Z-disk, with smaller amounts in the I-band and in the A-band area (Goll et al., 1999). It is generally accepted that the calpains are predominately cytosolic. However, calpain and its inhibitor calpastatin have been shown to be associated with cellular membranes (Brandt et al., 1992; Mellgren, 1987; Seiler et al., 1984). Mammalian skeletal muscle generally contains equal amounts of μ- and m- calpain (Goll et al., 1999), however, amounts of μ- and m- calpain vary in one tissue from another. The nomenclature for μ- and m- calpain is derived from the amount of Ca^{2+} that is required to activate the proteases in vitro (Suzuki, 1991). In order to activate μ-calpain to half maximal activity in vitro, 5-50 μM

Ca^{2+} is required (Edmunds, Nagainis, Sathe, Thomson, and Goll, 1991), while m-calpain requires 300-1000 μM Ca^{2+} for half maximal activity (Goll, 1991). As intracellular calcium concentrations fluctuate normally at submicromolar levels, μ-calpain is more likely to be functional in cells under physiological conditions.

Structure of the calpains. Both μ- and m-calpain are composed of two heterodimeric subunits, an isoform-specific catalytic 80 kDa and a common regulatory 28 kDa subunit (Goll, 1991). The large subunit of μ-calpain consists of 714 amino acids (Aoki et al., 1986) while the small subunit is comprised of 268 amino acids (Ohno, Emori and Suzuki, 1986). Although the 80 kDa subunit in μ- and m-calpain originated from two different gene products, a 50-60% amino acid homology exists (Goll, Dayton, Singh and Robson, 1991). The 28 kDa subunit is identical in both calpains as it originates from the same gene product (Goll, 1991). Considered "classical" calpains, μ- and m-calpain contain six domains in their heterodimeric subunits that are important in the activation and function of the polypeptide.

The large subunit is considered to be the catalytic subunit, and consists of four domains (I to IV) and a N-terminal anchor (Hosfield, Elce, Davies and Jia, 1999). Each domain is unique in its characteristics. The N-terminal, or anchor, region consists of amino acid residues 1-19 in m-calpain (Hosfield et al., 1999) and acts to stabilize the circular domain arrangement of the structure (Khorchid and Ikura, 2002). The anchor region of the large subunit along with domain V of the small subunit are suggested to connect the two units (Reverter et al., 2001; Strobl et al., 2000). The anchor region is currently considered not as a separate domain but as a part of domain I (Goll et al., 2003). Although the function is unclear, domain I contains the autolysis cleavage site for calpain (Goll, 1991). Domain II is the cysteine protease domain and contains the catalytic site (Khorchid and Ikura, 2002). It

can be divided into two subdomains, IIa and IIb (Strobl et al., 2000). The subdomains contain a catalytic triad of a Cys105, His262, and an Asn286 residue that resembles triads found in other cysteine proteases such as papain, cathepsins B and H, and bromelain (Khorchid and Ikura, 2002). These residues are responsible for the protease activity of calpain (Arthur, Gauthier and Elce, 1995). However, the similarity to other proteases is limited to the area of the active site residues. Between domain II and III, there was previously thought to exist a sixth EF-hand motif (Andresen, Tom and Strand, 1991). However, Reverter and others (2001) found that rather than possessing a sixth EF-hand motif, the current calpain structure exhibits a helix-loop-strand-helix different from the typical EF-hand folds, which exists as a part of the subdomain IIb. Domain III links the catalytic domain to the Ca^{2+} binding domain and contains an acidic loop that is suggested to be involved in mediating the Ca^{2+} activation signal to the protease (Hosfield et al., 1999; Strobl et al., 2000; Vilei et al., 1997). It has been shown to bind phospholipids in a Ca^{2+}-dependent manner, which may be important in reducing the concentration of calcium required in vivo for activation (Hosfield, Moldoveanu, Davies, Elce and Jia, 2001; Saido, Shibata, Takenawa, Murofushi and Suzuki, 1992; Tompa, Emori, Sorimachi, Suzuki and Friedrich, 2001). Through binding phospholipids, calpain may also be able to become associated with membrane lipids. Additionally, it is domain III that is proposed to interact with the endogenous inhibitor of calpain, calpastatin. Domain IV, the C-terminal domain, is α-helical and contains an amino acid sequence that is homologous to calmodulin (Goll, 1991). It also contains five EF-hand structures that play a role in Ca^{2+} binding and can be divided into subdomains EF-1 to EF-5 (Kinbura et al., 1998). EF-1 and EF-2 are paired, as are EF-3 and EF-4, while EF-5 is necessary for dimerization (Kinbura et al., 1998).

The small 28 kDa regulatory subunit is comprised of domain V and VI and is thought to be involved in the regulation of calcium sensitivity. Domain V is the N-terminus and is glycine rich which contributes to its hydrophobic properties. It is suggested that domain V is responsible for binding phospholipids. A polyproline link connects domain V to the C-terminal domain VI. The calmodulin-like domain VI is homologous to domain IV of the large subunit as it contains five EF hand motifs (EF1-EF5), four of which are involved in binding calcium (Hosfield et al, 1999) and a newly identified fifth motif involved in dimerization but not involved in calcium binding. Only EF1 and EF2 display an "open" conformation similar to that found in calmodulin, while the EF3 and EF4 show a confirmation similar to the two C-terminal EF-hand structures in troponin C and maintain a "closed" conformation when calcium is bound (Johnson and Guttman, 1997). The fifth EF-hand structure is in a closed conformation, and thus does not bind calcium. However, this structure is involved in an unique interaction with the fifth EF-hand motif of domain IV of the large subunit that results in heterodimeric binding between the two units (Khorchid and Ikura, 2002).

Mode of calpain activation. There are several hypotheses on how the calpains are activated. Suzuki, Sorimachi, Yoshizwa, Kinbura and Ishiura (1995) proposed that the first step in activation is that the heterodimeric structure undergoes a Ca^{2+}-dependent translocation to the cell membrane. In this hypothesis, dissociation does not occur initially as the small subunit is responsible for membrane interaction by its hydrophobic NH_2 terminus (Suzuki et al., 1995). At the membrane, phospholipids would be able to activate the calpains. Autolysis would then occur at the cell membrane as phospholipids would decrease the Ca^{2+} requirement of calpain (Suzuki et al., 1995).

An alternate hypothesis suggests that activation can be achieved by a Ca^{2+}-dependent dissociation of the small from the large subunit (Yoshizawa, Sorimachi, Tomioka, Ishiura and Suzuki, 1995). The dissociated 80 kDa is fully active and exhibits an increased Ca^{2+} sensitivity over the activated form of calpain. This hypothesis suggests that the 30 kDa subunit has a stabilizing rather than an activating effect on the catalytic 80 kDa subunit (Yoshizawa et al., 1995).

Effect of pH on calpain activity. Calpain activity can be affected by several factors. Temperature and pH (Koohmaraie, 1992a; Dransfield, 1994; Huff-Lonergan et al., 1996; Hwang and Thompson, 2001; Rowe et al., 2001b), ionic strength (Tan, Goll, and Otsuka, 1988; Kendall, Koohmaraie, Arbona, Williams, and Young, 1993; Elce, Hegadorn, and Arthur, 1997; Geesink and Koohmaraie, 1999; Geesink and Koohmaraie, 2000) and oxidation (Guttman, Elce, Bell, Isbell, and Johnson, 1997; Guttman and Johnson, 1998) have been shown to impact activity.

A low pH is expected to reduce calpain activity. Optimum pH for activity of either μ- or m-calpain or their autolyzed products is 7.4 – 7.6 (Edmunds et al., 1991). However, postmortem conditions of meat are at a pH of approximately 5.5. Thus, it would be expected that the calpains would lose activity with a decreasing pH and would not be fully active during times postmortem. Koohmaraie and others (1986) showed that in conditions similar to postmortem aging with a pH 5.5 to 5.8 and at 5°C, μ-calpain retained 24-28% of its maximum activity at pH 7.5 and at 25°C, which suggests that a sample with a higher pH would have greater calpain activity. Additionally, Koohmaraie (1992a) observed that when substrates were absent, an accelerated rate of μ-calpain autolysis occurred as the pH decreased from 7.0, 6.2, to 5.8. Claeys, De Smet, Demeyer, Geers and Buys (2001)

supported these findings when they observed a lower μ-calpain activity in the a genetic line with a faster rate of pH decline than the genetic line with a slower rate of pH decline. Rowe and others (2001a) observed that the porcine longissimus dorsi that had the lowest pH at 2 hours postmortem also had the furthest extent of autolysis and the least detectable activity at 24 hours postmortem. An absence of activity was found at 72 hours postmortem in the described samples. However, an earlier degradation of troponin-T and desmin was observed in the pork samples that had a low pH at 2 hours postmortem (Rowe et al., 2001a). This could suggest that a lower pH could accelerate the activity of calpain, but cause it to lose activity at a faster rate. A study by Dodge (2002) supports the hypothesis that pH can affect calpain activity. It was found in this study that pork muscles with a low pH at 45 minutes postmortem had the greatest extent of autolysis. Additionally, it was shown that the pork muscle sample that had the faster rate of pH decline, accelerated protein degradation was also observed.

O'Halloran and others (1997) also evaluated the calpain system in relationship to fast glycolysing muscle. The authors used bovine longissimus dorsi muscles that were selected according to their rate of glycolysis. They observed that muscles with a faster rate of pH decline had increased μ-calpain activity early postmortem (O'Halloran et al., 1997). Additionally, increased proteolysis indicated by the appearance of degradation products of proteins involved in water-holding capacity and tenderness at earlier times postmortem was found in muscles with a faster rate of pH decline (O'Halloran et al., 1997). Using transmission electron microscopy, greater ultrastructural breakdown of the myofibril early postmortem was also found in fast glycolysing muscle (O'Halloran et al., 1997). These

results suggest that increased proteolysis and breakdown of the myofibril in muscles with a fast rate of pH decline may be due to increased calpain activity.

Regulation of Calpain. Regulation is a very important aspect relative to the calpain system. Without an intricate strategy for regulation of calpain proteolytic activity, the protease could cleave many intracellular proteins involved in signaling and structure, thus, a state of proteolytic inactivation must be maintained by the calpains most of the time. Physiologically, the calpains do not simply degrade substrates into small peptides and amino acids, but rather modulates their properties by cleaving in a restricted manner, leaving large polypeptide fragments (Suzuki and Sorimachi, 1998). In order for the calpains to be effective and limited in their scope, activity must be tightly regulated. Current research provides evidence that the calpain system is post-transcriptionally regulated by many mechanisms including calcium requirements, calpastatin activity, intracellular distribution in vivo, autoproteolytic cleavage, phosphorylation and subsite specificity of the calpains (Goll et al., 2003).

Calpain Autolysis. Autolysis is an important method of regulation for the calpain system. The calpains have an ability to undergo autolysis, or self proteolytic activity, when in the presence of Ca^{2+} (Suzuki et al., 1981a; Suzuki, Tusji, Kubata, Kimura and Imajori, 1981b; Hathaway, Werth and Haeberle, 1982). In μ-calpain, this mechanism of self-proteolysis results in the removal of a 14 amino acid sequence from the N-terminus which forms an active 78 kDa intermediate product (Johnson and Guttman, 1997). In m-calpain, the 78 kDa intermediate is formed from the removal of an 9 amino acid sequence from the N-terminus (Johnson and Guttman, 1997). An additional 12 amino acids in μ-calpain and 10 amino acids in m-calpain is then removed from the N-terminal end of the 78 kDa subunit to

form a 76 kDa subunit in the active state (Johnson and Guttman, 1997; Zimmerman and Schlapfer, 1991). Autolysis of the small regulatory subunit results in the loss of 91 amino acids from the N-terminus (the Gly-rich region) to form a 18 kDa subunit in both μ- and m-calpain (Goll et al, 2003). Autolysis of the small subunit is suggested to occur as a series of amino acid removals rather than a simultaneous removal, with a 26 amino acids first removed from the NH_2-terminal end, followed by an additional 37 amino acids, and lastly 28 amino acids are removed in order to produce the 18 kDa fragment (McClelland, Lash and Hathaway, 1989). Autolysis of the 28 kDa subunit in m-calpain occurs more rapidly than the 80 kDa subunit, however, in μ-calpain, autolysis of the large subunit occurs at the same or faster rate than the small subunit (Zimmerman and Schlaepfer, 1991). It has been suggested that autolysis results in separation of the large and small subunits. Kitagaki and others (2000) showed through 2-dimensional electrophoresis that autolysis results in the two subunits completely and irreversibly dissociating from one another.

Autolysis of the calpains is related to Ca^{2+} concentration. Suzuki and others (1981a) first described that chicken calpain undergoes autolysis rapidly in the presence of sufficient calcium. However, in order to initiate autolysis of m-calpain, the calcium concentration must be increased to nearly 100 μM which is higher than the free Ca^{2+} concentration physiologically found in the cell cytosol (Suzuki et al., 1981a). It is important to note that the concentration required for autolysis is similar or greater than the concentration required for proteolytic activity (Zimmerman and Schlaepfer, 1991) and more calcium is required for autolysis than activity. Even though other conditions required for autolysis are met in vivo, autolysis can not occur without a Ca^{2+} concentration increase. Although it is not thoroughly understood, autolysis does occur under physiological conditions (Saido, Suzuki, Tanoue and

Suzuki, 1993). It is possible that a local increase may initiate autolysis and thus the process would be able to occur at specific sites in the cell where the calcium concentration is high (Mellgren, Repetti, Muck and Easly, 1982; Suzuki et al., 1981b). Additionally, calpain autolysis lowers the calcium requirement for activation, but the exact manner in which autolysis lowers the calcium requirement is unknown. Calpain sensitivity to Ca^{2+} ions is enhanced by autolysis and the product obtained by autolysis is active at physiological concentrations of Ca^{2+} (Suzuki et al., 1981b). Studies have shown that the calcium requirement for half maximal activity of m-calpain was lowered from 400-800 mM to 300 μM when the 80 kDa autolyzed to the 76 kDa subunit and the 30 kDa autolyzed to the 18 kDa subunit (Hathaway et al., 1982; Mellgren et al., 1982). In a study done by Suzuki and others (1981a, b), when incubated in vitro with 1 mM free Ca^{2+}, m-calpain autolyzed from 80 to 79 kDa and reduced the Ca^{2+} concentration required for half-maximal activity from approximately 400 μM to 30-50 μM. The rate of autolysis is also very dependant on Ca^{2+} concentration. Cottin, Thomson, Sathe, Szpacenko and Goll (2001) showed that autolysis of μ-calpain was rapid at 5 mM Ca^{2+}, less rapid at 2 mM Ca^{2+}, and the least rapid at 100 μM Ca^{2+}.

Both μ- and m-calpain appear active in vivo at physiological calcium concentrations of 100-300 nM (Goll, Thompson, Taylor and Christiansen, 1992). This activity may indicate that other factors such as binding of phospholipids, DNA, and activator proteins may play a role in activation and could explain possible mechanisms for calpain activation at in vivo calcium levels (Saido et al., 1992). Coolican and Hathaway (1984) showed that calpain association with the phospholipids phosphatidylinositol and dioleoylglycerol resulted in eightfold lower calcium concentrations required for autolysis of m-calpain in bovine aortic

smooth muscle. Later studies conducted supported that incubation with phospholipids in vitro lowers the Ca^{2+} concentration required for half-maximal rate of μ- or m-calpain autolysis by three-to tenfold (Cong, Goll, Peterson, and Kapprell, 1989; Pontremoli et al., 1985; Saido et al., 1992). Additionally, it was shown that activator proteins can sensitize calpains to calcium up to 100 fold (Pontremoli, Sparatore, Melloni, Michetti and Horecker, 1984; Salamino, DeTullio, Viotti, Mellgren and Pontremoli, 1993), and DNA can sensitize calpain to calcium by 100 fold (Mellgren, 1991). Although the actual significance is still unclear, it may be possible that binding to phospholipids, DNA, and/or activation proteins may lower the required calcium concentration for calpain activity.

Autolysis was once hypothesized to be required for activity. Because the calcium requirement for autolysis is similar or slightly greater than the requirement for activity, in vitro assays of proteolysis appears to parallel autolysis (Hathaway et al., 1982; Inomata, Hayashi, Nakumura, Imahori and Kawashima, 1985; Inomata, Imahori and Kawashima 1986; Coolican, Haiech and Hathaway, 1986). This led to the hypothesis that the unautolyzed 80 kDa/30 kDa molecule acted as a proenzyme until autolysis occurred (Mellgren, 1987; Suzuki et al., 1987; Suzuki, 1991). However, Elce and others (1997) showed that the unautolyzed 80 kDa subunit of m-calpain (after it had been altered at the autolytic cleavage site so it could not autolyze), was still capable of proteolytic activity when incubated with EDTA and $CaCl_2$. Cottin, Poussard, Desmazes, Geiorgescauld and Ducastaing (1991) found that calpain activation requires less free Ca^{2+} than the proteolysis of a digestible substrate, which indicates that proteolysis is subsequent to activation. Additionally, other studies suggest that autolysis lowers the Ca^{2+} concentration required for proteolytic activity closer to physiological range of calcium found in vivo. (Cottin et al., 1991; Zimmerman and

Schlaepfer, 1991; Saido et al., 1994; Baki, Tompa, Alexa, Molnar and Friedrich, 1996) which suggests that activation proceeds proteolytic activity. Guttman and others (1997) showed that oxidation inhibits proteolytic activity but does not prevent autolysis, thus the two events must be separate. Consequently, it does not seem likely that the calpains are proenzymes that require autolysis for activation, but the presence of autolysis may be able to be used as an indicator that the calpains have been active in a cell (Cong et al., 1989; Cottin et al., 2001; Elce et al., 1997).

Research has indicated that autolysis does play an important role in the function of the calpain system. However, the exact physiological significance remains unclear and further study is needed in order to probe deeper into the mechanism of calpain autolysis.

Calpastatin as a calpain inhibitor. Calpastatin, the naturally occurring inhibitor of the μ- and m-calpain, is ubiquitously expressed in various cells and is believed to influence calpain activity (Ouali and Talmant, 1990; Geesink and Koohmaraie, 1999; Huff-Lonergan and Lonergan, 1999). Calpastatin is generally localized in the same cellular region as calpain, although the protein is generally found at higher concentrations within the cell than μ- and m-calpain (Suzuki et al., 1987). Calpain and calpastatin are found together in the sarcolemma with the adjacent cytoplasm, the myofibrils, the mitochondria, and the nuclei. The inhibition activity of calpastatin is specific for calpain and it does not inhibit other cysteine proteinases such as papain and cathepsin B and L (Kapprell and Goll, 1989). Inactivation of calpastatin with the calpains is reversible when the proteins dissociate from one another (Kapprell and Goll, 1989). Calpastatin has been shown to play a major role in the regulation of proteolysis that occurs as a result of the calpains (Huff-Lonergan and

Lonergan, 1999), as well as facilitating Ca^{2+} influx by restoring run-down membrane Ca^{2+} channel activity (Hao et al., 2000).

The primary structure of the 110 kDa calpastatin consists of an unique alkaline N-terminal region known as domain L followed by four repeating subunits (domain I-IV) that contain homologous amino acid sequences of approximately 140 residues; the residues in the four repeating domains are further grouped into three regions A, B, and C (Maki et al., 1991; Melloni, Salamino and Sparatore, 1992; Takano et al., 1988). Region B are important for the Ca^{2+}-dependent inhibition of calpain while regions A and C aid in the binding of region B to calpain (Maki et al., 1988; Kawasaki, Emori, Imajoh, Minami, and Suzuki, 1989). When calcium is present, domains I-IV of calpastatin can each inhibit the proteolytic activity of μ- and m-calpain; each domain is a functional unit (Kawasaki and Kawashima, 1996; Maki et al., 1987). Domain L possesses no inhibitory activity but instead is suggested to restore run down membrane Ca^{2+} channel activity and can therefore facilitate the Ca^{2+} influx (Hao et al., 2000). Although the precise mechanism for regulation is unknown, calpastatin inhibits calpains by binding regions A and C to domain IV and VI of μ-calpain, respectively (Nishimura and Goll, 1991). Additionally, the conserved regions B cover the catalytic site of domain II in calpain and are the inhibitory site of calpastatin.

Calpastatin can not inhibit the initial autolysis step of the 80 kDa subunit, but it can inhibit calpain autolysis from 78 kDa to 76 kDa (Johnson and Guttman, 1997; Zimmerman and Schlaepfer, 1991). Inhibition of calpain by calpastatin is calcium dependent; Ca^{2+} is required for calpastatin to bind to and inhibit the calpains (Kapprell and Goll, 1989). Calcium must bind to calpain in order for a conformational change to occur on the calpain molecule that subsequently allows calpastatin to bind to calpain. Less calcium is required

for calpastatin to bind to the calpains than required by the calpains for activity (Kapprell and Goll, 1989). Additionally, less Ca^{2+} is required for half maximal binding to calpastatin for activity in autolyzed μ-calpain than in unautolyzed μ-calpain (Kapprell and Goll, 1989). The inhibition effects on calpain are reversible when Ca^{2+} chelators are added (Maki, Hatanaka, Tokano, and Murachi, 1990), thus the amount of calpastatin in cells relative to the amount of calpain is an important factor in order to determine the extent of calpain activity. As no current research supports the idea that calpastatin binds calcium, it is currently believed that the calcium dependency of the interaction between calpain and calpastatin must arise from the calpain molecule and its conformational change that occurs when Ca^{2+} is bound (Goll et al., 2003).

Calpastatin has been shown to play a large role in the regulation of postmortem proteolysis (Huff-Lonergan and Lonergan, 1999). Increased activity levels of calpastatin has been shown to limit both the rate and the extent of proteolysis by μ-calpain in bovine myofibrils, but calpastatin was not able to completely inhibit proteolysis (Geesink and Koohmaraie, 1999). Lonergan and others (2001) showed that pigs selected for lean growth had significantly higher levels of calpastatin activity than the control line. The lean growth line also had significantly less troponin-T degradation, which suggests that the increased calpastatin activity effectively decreased proteolytic activity (Lonergan et al., 2001).

Calpastatin is affected by pH. As the pH in muscle is lowered, particularly below 6.4, calpastatin inhibitory activity decreases. O'Halloran and others (1997) revealed lower calpastatin levels in fast glycolysing longissimus muscles from heifers at 3 h postmortem. Thus, the authors concluded that calpastatin regulation could be contributing to improved

tenderness of muscles with a faster rate of pH decline versus muscles with a slower rate of decline (O'Halloran et al., 1997).

Calpastatin not only inhibits calpain, but is also a substrate of calpain (Murachi, Tanaka, Hatanaka, and Murakami, 1981; Goll et al., 1983). Calpastatin is cleaved by calpain in the interdomain regions which in turn generates inhibitory peptides and can then inhibit more than one molecule of calpain. It has been suggested that degradation of calpastatin by calpain does not result in the loss of calpastatin's ability to inhibit the calpains (Goll et al., 1983; Mellgren, Mericle, and Lane, 1986; Mellgren and Carr, 1983). However, Doumit and Koohmaraie (1999) disagreed with this hypothesis and showed that degradation of calpastatin by μ-calpain did reduce inhibitory activity. Reduced inhibition as postmortem storage progressed could greatly affect regulation of proteolysis and which could thus affect water-holding capacity.

Role of the calpain system. The precise physiological function of the calpain system in vivo is not exactly known. However, the ubiquitous nature and constant expression of μ- and m-calpain suggest that they contribute to basic and essential cellular processes including signal transduction, apoptosis, cell cycle regulation and cytoskeletal remodeling (Molinari and Carafoli, 1997; Sorimachi, Ishiura and Suzuki, 1997; Carafoli and Molinari, 1998; Ono, Sorimachi and Suzuki, 1998; Hosfield et al., 2001). Calpain has been shown to be critically important for normal growth as transgenic mice that lack the calpain regulatory subunit and do not express any calpain activity die during embryonic development (Arthur, Elce, Hegadorn, Williams and Greer, 2000).

The calpain system has been implicated in several pathological conditions such as muscular dystrophy, cardiac and cerebral ischemia, platelet aggregation, neurodegenerative

diseases, rheumatoid arthritis, cataract formation, and Alzheimer's disease (Hosfield et al., 2001; Khorchid and Ikura, 2002). It is suggested that cellular stress triggers altered Ca^{2+} homeostasis which results in an elevated Ca^{2+} concentration (Hosfield et al., 2001). This in turn then causes an unregulated over-activation of calpain as well as mutations (Hosfield et al., 2001).

In addition to their roles in living tissue, there is substantial evidence that the calpains have an instrumental role in postmortem proteolysis of key myofibrillar proteins (Koohmaraie, 1992b; Ouali, 1992; Huff-Lonergan et al., 1996; Koohmaraie, 1996). Calpain has been shown to degrade desmin (O'Shea, Robson, Huiatt, Hartzer, and Stromer, 1979), troponin T (Ho, Stromer, and Robson, 1994), titin (Suzuki, Kim, and Ikeuchi, 1996), nebulin (Taylor, Geesink, Thompson, Koohmaraie, and Goll, 1995), talin (Hemmings et al., 1996) and vinculin (Taylor et al., 1995). However, calpain does not degrade the main myofibrillar proteins actin (Goll et al., 1991) or myosin (Pemrick and Grebanau, 1984), although evidence suggests that calpain is able to "nibble" the N-terminal end of the 200 kDa myosin polypeptide in order to produce several polypeptide fragments of 150-180 kDa (Goll et al., 1999). The inability of calpain to proteolyze certain proteins shows that they have a limited and very specific substrate specificity. Additionally, collagen is not degraded by calpain (Goll et al., 1999).

Calpain degradation of the myofibril. The calpain system has been shown to remove the Z-lines from skeletal muscle myofibrils when calpain is added directly to the myofibrils. Initially, incubation with purified calpain results in a loss of the N-line structures on either side of the Z-disk which is accompanied by a reduced density of the Z-disk. Gradually, there is a complete loss of density of the Z-disk (Goll et al., 1991). The thin filaments are also

affected, but the thick filaments are unchanged. However, this does not occur identically in naturally aged meat. Taylor and others (1995) showed that as the calpains are the main proteolytic enzymes in skeletal muscle during the first 72 to 96 hrs. postmortem, direct Z-disk degradation did not occur. Rather, in postmortem muscle, the calpains cause proteolysis of the costameres and the intermyofibrillar linkages which result in a weakening of the thin filament/Z-disk interaction (Taylor et al., 1995).

The calpains are also responsible for degradation of the costameres. Costameres are important cytoskeletal elements which are located in the junctions at the I-band between the sarcolemma adjacent myofibrils and the cell membrane and are responsible for linking the myofibrils to the sarcolemma as well as to each other (Stromer, 1998). Several important proteins such as desmin, talin and vinculin are components of the costamere that lie beneath the cell membrane (Robson et al., 1997). Post-mortem proteolysis of the costameric proteins talin and vinculin results in a loss of structural stability of the muscle fiber (Wheeler and Koohmaraie, 1994) and may be implicated in variations in water-holding capacity (Morrison, Mielche and Purslow, 1998; Kristensen and Purslow, 2001).

During the postmortem storage of meat, cytoskeletal proteins are degraded (Taylor et al., 1995; Ho, Stromer and Robson, 1996; Koohmaraie, Shackelford, Wheeler, Lonergan and Doumit, 1995). This degradation has been shown to be related to meat tenderness (Huff-Lonergan, Parrish and Robson, 1995; Taylor et al., 1995; Morrison et al., 1998). More recently, degradation of the costameric proteins talin and vinculin has also been shown to be related to water holding capacity. Kristensen and Purslow (2001) showed that the degradation of the costameric proteins talin and vinculin coincided with an improvement in water holding capacity. Degradation of the costameres may weaken muscle fiber structure

and can create intracellular gaps when the sarcolemma detaches from the Z-lines of the myofibrils (Taylor et al., 1995). Thus, the linkage between the rigor induced lateral shrinkage of myofibrils and the shrinkage of the entire muscle fiber would be reduced or eliminated (Kristensen and Purslow, 2001). With the degradation of the costameric proteins, the cytoskeletal connections are no longer intact and cell shrinkage can not continue. With the force (shrinking) that causes cellular water to flow into the extracellular space removed, intracellular water could be retained in the cell. Additionally, water that has been previously expelled can then flow back into the muscle cell. This could result in an increase in water holding capacity during postmortem storage. Taylor et al. (1995) found that the costameres were degraded during the first 24 to 72 hours postmortem.

Talin and vinculin have been shown to be excellent substrates of calpain. The costameres are located below the surface of the muscle cell where they would be immediately exposed to any extracellular Ca^{2+} leaking into the muscle cell due to damage or weakening of the sarcolemma during postmortem storage (Jeacocke, 1993). In turn, the calcium may activate calpain which then degrade the costameric proteins.

Talin is one of a number of interacting proteins which link the cytoplasmic domains of integrins to the actin cytoskeleton. Talin has been shown to be rapidly cleaved to produce a 190 kDa C-terminal and 47 kDa N-terminal fragment when incubated in vitro with calpain (Rees, Ades, Singer and Hynes, 1990). The C-terminal fragment is composed of a large number of alanine-rich repeats of 34 amino acids in length (McLachlan, Stewart, Hynes and Rees, 1994) and several binding sites for vinculin (Gilmore et al., 1993) and actin (Hemmings et al., 1996).

Vinculin, another important protein of the skeletal muscle costamere, is involved in the linkage of actin bundles to the cell membranes. Vinculin is a 130 kDa protein that can be degraded by calpain in vitro to form a 90kDa fragment (Evans, Robson and Stromer, 1984; Taylor et al., 1995). Vinculin is very susceptible to degradation in postmortem muscle and vinculin degradation begins in the first 24 hours postmortem and is almost complete by 72 hours (Taylor et al., 1995).

Calpain cleavage of the RyR and SERCA. Calpain has been shown to be able to degrade membrane channel Ca^{2+} regulating proteins such as the SERCA pump and the RyR. Shoshan-Barmatz and others (1994) showed that cleavage of the RyR by calpain did not affect RyR binding activity, but does stimulate Ca^{2+} release. Thus, this degradation could allow Ca^{2+} leakage which could thus elevate the Ca^{2+} ion concentration enough to further activate the calpains. Further studies are required to evaluate this hypothesis.

Calpain initially cleaves the 565 kDa RyR monomer into peptides of 160 and 410 kDa, which is subsequently cleaved into 70 and 340 kDa (Brandt, Caswell, Brandt, Brew and Mellgren, 1992). The 340 kDa peptide is then subject to further cleavage at higher calpain concentrations and is finally cleaved to 140 and 200 kDa products (Brandt et al., 1992). Calpain appears to cleave the RyR in an ordered sequence of susceptible sites; the first site must be cleaved before the second becomes exposed (Brandt et al., 1992). Cleavage of the ryanodine receptor is pH dependent; the maximum cleavage of the RyR by μ-calpain was observed between pH 7.0 and 7.5 (Shoshan-Barmatz, et al., 1994), which is substantially higher than the 5.5-5.7 pH found in postmortem tissue. Additionally, the presence of ATP and high NaCl concentrations decreased RyR cleavage by the calpains (Shoshan-Barmatz et al., 1994).

Wang and others (1989) showed that calpain cleaves its substrate proteins at or near calmodulin binding sites. Calmodulin binding proteins are recognized by their PEDST (proline, glutamic acid, aspartic acid, serine, threonine rich) protease cleavage sequences (Wang et al., 1989). These substrates are usually cleaved near calmodulin binding sites and, once cleaved at these sites, further degradation by calpain is altered or inhibited (Brandt et al., 1992; Wang et al., 1989). Brandt and others (1992) hypothesized that the RyR was a PEDST-type calpain substrate and showed that the Ca^{2+}-releasing protein has eight PEDST sequences. The fact that the RyR appears to be a PEDST-type calpain substrate implies that calpain is responsible for the normal turnover rate of the RyR in vivo (Brandt et al., 1992).

Calpain is predominately cytosolic; however, association of the calpains and calpastatin with cellular membranes has been suggested (Brandt et al., 1992, Mellgren, 1987; Mellgren, Lane and Kakar, 1987). Association with the junctional triads has been reported (Brandt et al., 1992) and immunoelectron-microscopic studies have shown calpain activity to be located at or near the T-tubule in rat skeletal muscle (Yoshimura et al., 1986). Rardon and others (1990) have shown that calpain treatment of junctional SR membranes increased the open probability of the Ca^{2+} release channel. This observation was in agreement with Shoshan-Barmatz et al., (1994) who noted an enhanced Ca^{2+} efflux from the sarcoplasmic reticulum at the site where the RyR was cleaved.

Calpain is closely regulated by the intracellular Ca^{2+} concentration. A small increase in intracellular Ca^{2+} could activate the membrane associated μ-calpain. This would cleave the RyR, thereby activating Ca^{2+} release. These initial events would lead to a further increase in the intracellular Ca^{2+} that would further increase proteolysis by μ- and m-calpain and lead to further proteolysis of muscle proteins.

Another protein important in calcium homeostasis and regulation is the sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) pump. This membrane associated protein can also be degraded by calpain. Zhao, Levin, Wein and Levin (1997) reported that experimental ischemia and partial outlet obstruction of the rabbit urinary bladder cause tissue ischemia and reperfusion injury that subsequently results in an increased intracellular Ca^{2+} concentration. This increase in Ca^{2+} concentration was followed by calpain activation and proteolysis of the SERCA pump, which resulted in reduced muscle function (Zhao et al., 1997).

Summary

This literature review addressed the topics necessary to provide support for the work in this thesis. By compiling studies previously reviewed, it is apparent that there is a definite relationship between pH decline, μ-calpain autolysis, and degradation of membrane proteins involved in calcium homeostasis. Thus, evidence exists to support the hypothesis that proteolytic processes in early postmortem pork influence pH decline and water-holding capacity.

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IMPACT OF PROTEOLYSIS ON pH DECLINE AND WATER-HOLDING CAPACITY OF FRESH PORK

A paper to be submitted to Meat Science

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Abstract

The objective of this study was to determine the relationship between pH decline, μ-calpain autolysis, proteolysis of the costameric protein vinculin, and degradation of membrane proteins involved in calcium homeostasis. Commercial hybrid pigs (n = 309) from two different genetic lines (lines 2 and 3) were harvested at a commercial facility. Measurements for pH were taken in the longissimus dorsi (LD) at 45 min, 3, 6, and 24 h postmortem (PM). In order to obtain animals with variability in pH measurements, two groups (high and low pH at 3 h PM) were selected within each line (line 3/ high average pH = 6.19, line 3/ low average pH = 5.41, line 2/ high average pH = 6.27, and line 2/ low average pH = 5.47). Percent drip loss after 24, 48, and 96 h storage was determined on the selected animals. Within each of the four groups, ten animals were selected to determine autolysis of μ-calpain and the extent of proteolysis of the skeletal muscle ryanodine receptor (RyR1), sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA2), and vinculin at 1 d, 48, 72 and 120 h PM. The line 2/high pH group had a higher ($P < 0.05$) pH than all other groups at 24 h PM. The line 2/high pH group also had a lower ($P < 0.01$) percent drip loss at 24 and 48 h storage in comparison to line 2/low and line 3/low pH groups. The line 2/high pH group also had

the least ($P < 0.05$) amount of drip at 96 h storage. Increased drip loss at 24, 48, and 96 h storage was correlated ($P < 0.05$) to decreased pH at 45 min, 3, 6, and 24 h PM. The line 3/low pH group had the highest ($P < 0.05$) L* value than all other groups. At 1 d postmortem, the high pH groups had increased ($P < 0.05$) μ-calpain autolysis compared to the low groups. Increased autolysis of μ-calpain was correlated ($P < 0.05$) to higher pH and decreased drip loss. Line 3 had more ($P < 0.05$) RyR1 degradation (band 2) than line 2 at 48 h PM. An increase in RyR1 degradation (band 2) was related to lower 6 h ($P < 0.05$) and 24 h ($P < 0.01$) pH and more drip loss at all times measured ($P < 0.01$). Increased proteolysis of intact RyR1 at 1 d and 48 h was correlated ($P < 0.01$) to increased μ-calpain autolysis. Increased proteolysis of SERCA2 was correlated ($P < 0.05$) with lower pH and increased drip loss. Decreased degradation ($P < 0.05$) of vinculin was found in line 2/low pH compared to both groups in line 3. Correlations revealed that samples with a higher early postmortem pH had increased ($P < 0.05$) degradation of vinculin. Also, increased degradation of vinculin at 48 h PM was correlated ($P < 0.05$) to increased μ-calpain autolysis at 1 d and 48 h PM. These results show that μ-calpain autolysis and proteolysis may be related to Ca^{2+} regulation and may affect quality attributes in pork such as water-holding capacity.

Key Words: Calpain, Ca^{2+} regulation, Pork, Proteolysis, Water-Holding Capacity

Introduction

Lack of consistently high quality pork has become a major obstacle standing in the way of increasing pork consumption. While consumption of pork has remained stable, consumers have increased their consumption of other products such as poultry, fish, and

protein alternatives from plant sources (National Pork Board, 2002/2003). As a result the industry has been negatively influenced by poor pork quality. Therefore, it is important that steps are taken to improve the quality of pork in order for the industry to remain competitive and profitable.

One of the most prevalent quality issues in the pork industry is unacceptably high drip loss in fresh products. High drip loss can result in lost product weight and thus lost financial value. Additionally, it impacts the appearance of the fresh product, consumer perception at the time of purchase, and the sensory properties of the cooked product. Biochemical factors such as accelerated pH decline and low ultimate pH are related to the development of low water-holding capacity and unacceptably high drip loss. A rapid pH decline and/or a low ultimate pH (5.0-5.2) can result in decreased water-holding capacity of fresh pork (Offer and Knight, 1988; Lonergan, Huff-Lonergan, Rowe, Kuhlers and Jungst, 2001). The factors that control the rate and extent of pH decline need to be resolved in order for the pork industry to improve water-holding capacity of fresh meat products.

Postmortem calcium regulation by the skeletal muscle ryanodine receptor (RyR1) and the sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) pump can affect the rate of pH decline. Pigs that have inherited a mutation in the ryanodine receptor/calcium release channel (halothane gene) are most likely to develop pale, soft and exudative (PSE) meat and are found to have the most severe drip loss (Lister, Gregory and Warriss, 1981). This mutation causes an impaired ability of the RyR1 to control calcium release into the sarcoplasm of the muscle cell, particularly under periods of stress. An accelerated calcium release can occur, which causes strong and rapid muscle contraction and subsequently an increase in the rate of muscle metabolism and in the rate of pH decline. Despite successful efforts to reduce the

occurrence of PSE pork by selectively breeding against the halothane gene, there is still immense variability in water-holding capacity (Purslow et al., 2001). Thus, as calcium is an important mediator of muscle metabolism, it is highly likely that other alterations in calcium regulation may explain some of the remaining variations in the rate of pH decline and water-holding capacity.

Modulation of the sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) pump may also influence poor pork quality. The SERCA pump is responsible for removing calcium from the sarcoplasm and returning it to the sarcoplasmic reticulum. This pump functions as long as ATP is available. Failure of the SERCA to remove Ca^{2+} from the sarcoplasm and return it to the sarcoplasmic reticulum, particularly during the early postmortem period, could result in an increased postmortem metabolism and a more rapid rate of pH decline.

The SERCA pumps are encoded by three genes, which, after differential splicing, give rise to at least seven different isoforms: SERCA1a/b, SERCA2a/b, and SERCA3a/b/c (Møller, Juul and leMaire, 1996; Wuytack, Dode, BabaAissa and Raeymaekers, 1995). SERCA1a is predominately found in adult fast-twitch skeletal muscle while SERCA1b exists in neonatal muscle (Brandl, Green, Korckzak and MacLennan, 1986; Brandl, deLeon, Martin and MacLennan, 1987). The isoform SERCA2a is expressed in cardiac muscle (MacLennan, Brandl, Korczack and Green, 1985), slow-twitch skeletal, fetal fast-twitch, and smooth muscle (Brandl et al., 1987) while SERCA2b is found in most all nucleated cell types (Lytton, Zarain-Herzberg, Periasamy and MacLennan, 1989). SERCA3a and 3b are found in specialized cells such as mast cells, platelets, lymphocytes, endothelial and epithelial cells (Wuytack et al., 1995). The porcine longissimus dorsi muscle contains fast (type IIa and

IIb) and slow (type I) fibers (Christensen, Henckel and Purslow, 2004), thus would be expected to contain isoforms SERCA1 and 2.

Two Ca^{2+}-dependent cysteine proteases (μ-and m-calpain), and their naturally occurring inhibitor (calpastatin), have been shown to play an instrumental role in postmortem proteolysis of proteins that influence pork quality (Koohmaraie, 1988). It is important to note that pH can impact the activation of μ- and m-calpain as well as the activity during proteolysis. Rowe, Huff-Lonergan and Lonergan (2001a) found that the rate of pH decline and ultimate pH influences calpain activity and autolysis, in turn affecting the rate of protein degradation. The RyR1 has been shown to be a substrate of calpain (Brandt, Caswell, Brandt, Brew and Mellgren, 1992; Iino, Takano-Ohmura, Kawana and Endo, 1992; Shevchenko, Feng, Varsanyi and Shoshan-Barmatz, 1998; Shoshan-Barmatz, Weil, Varsanyi and Heilmeyer, 1994). The SERCA pump has also been shown to undergo proteolysis when incubated with calpain (Purintrapiban, Wang and Fosberg, 2001; Seiler, Wegener, Whang, Hathaway and Jones, 1984; Wang, Roufogalis and Villalobo, 1988a and 1988b; Zhao, Levin, Wein, and Levin, 1997). It is possible that degradation of the SERCA pump by calpain early postmortem could destabilize Ca^{2+} regulation by decreasing the pump's ability to remove calcium from the sarcoplasm (Zhao et al., 1997). An impairment of the RyR1 could stimulate Ca^{2+} entry into the cell (Iino et al., 1992). In both situations, the subsequent higher Ca^{2+} concentrations in the sarcoplasm could cause a faster rate of anaerobic metabolism and an accelerated pH decline. Alteration of the RyR1 and SERCA pump that would cause the proteins to decrease their Ca^{2+} regulating functions (release and uptake, respectively) may also affect the calpains. A moderate increase in sarcoplasmic Ca^{2+} could provide enough

calcium to activate μ-calpain. Thus, it is important to characterize the relationship between the degradation of the proteins involved in calcium homeostasis and the calpain system.

The costameres are important cytoskeletal elements that are located in the junctions at the Z-line between the sarcolemma adjacent myofibrils and the cell membrane. Costameres are responsible for linking the myofibrils to the sarcolemma as well as to each other (Stromer, 1998). It is possible that degradation of costameres may weaken muscle fiber structure and create intracellular gaps when the sarcolemma detaches from the Z-lines of the myofibrils (Taylor, Geesink, Thompson, Koohmaraie and Goll, 1995). Thus, the rigor induced lateral shrinkage of myofibrils would be less likely to be translated into shrinkage of the entire muscle fiber (Kristensen and Purslow, 2001). Intracellular water could be retained within the cell and previously expelled water could flow back into the muscle cell, resulting in improved water-holding capacity during postmortem storage.

The costameres are located below the surface of the muscle cell where they would be immediately exposed to any extracellular Ca^{2+} leaking into the muscle cell due to damage or weakening of the sarcolemma during postmortem storage (Jeacocke, 1993). In turn, the calcium may activate calpain which then degrades the costameric proteins. The costameric protein vinculin, a substrate of calpain (Evans, Robson and Stromer, 1984; Taylor et al., 1995), has been shown to be related to water-holding capacity. Kristensen and Purslow (2001) found that the degradation of the costameric protein vinculin coincided with an improvement in water-holding capacity.

The objective of this study was to determine the relationship between pH decline, μ-calpain autolysis, proteolysis of the costameric protein vinculin, and degradation of membrane proteins involved in calcium homeostasis. It was hypothesized that proteolytic

processes in early postmortem pork influence pH decline and water-holding capacity. In order to study these processes at different pH measurements, pigs from two different genetic lines were evaluated and animals within each line that varied widely in early pH (high vs. low) were selected for this study. Degradation of the RyR1 and SERCA2 by the calpain system could destabilize calcium regulation in early postmortem muscle and may be involved in more rapid pH decline. Additionally, alteration of the SERCA pump and RyR1 could decrease their Ca^{2+} regulating ability and thus promote an increased Ca^{2+} concentration in the cell sarcoplasm. This Ca^{2+} increase could be sufficient to meet the requirements needed for the activation of μ-calpain. In turn, this could promote the subsequent proteolysis of proteins involved in water-holding capacity.

Materials and Methods

Animals. Three-hundred and nine commercial hybrid pigs from two different genetic lines (Line 2 and Line 3) were used in this study. Both lines are free of the halothane and RN^- gene.

The pigs were harvested using humane procedures at a commercial slaughter facility. Measurements for pH (Mettler-Toledo glass tipped probe, Mettler-Toledo Process Analytical Inc., Wilmington, MA) were taken at 45 min, 3 h, and 6 h postmortem in the longissimus dorsi (LD) on the right side of the carcass at the last rib. The 24 h pH was taken in the center of the longissimus dorsi after its removal from the carcass. At 45 min and 3 h postmortem, pH was measured on three hundred and nine pigs, with one hundred and forty-nine animals from line 3 and one hundred and sixty animals from line 2 measured. To determine which pigs had substantial differences in pH, the 3 h postmortem pH measurements were evaluated.

Based on this data, groups of pigs with longissimus muscles that had relatively high and low early pH measurements were defined within each genetic line. The two groups were classified into low (pH < 5.7 at 3 h) and high (pH > 6.0 at 3 h) pH groups by using the 3 h postmortem pH data. From this data, twenty-seven animals in the low pH group and twenty-six animals from the high pH group for line 3 were selected and twenty-seven pigs in the low pH group and twenty-seven pigs in the high pH group for line 2 were selected. The 6 h and 24 h pH data were taken on the one hundred and seven selected animals from both genetic lines.

At 24 h postmortem, the loins were removed from the left side of each carcass and color (L* - muscle lightness; a* - muscle redness; b* - muscle yellowness) data was collected using a Minolta Colorimeter (CR-310, Minolta Camera Co., Ramsey, NJ), which was calibrated according to manufacturer's directions (settings: D65 illuminate, 10° observer). Measurements for 24 h pH were also taken at this time. The boneless loins were then vacuum packaged and immediately transported to Iowa State University, Ames, Iowa.

Drip Loss Analysis. After transport to the Iowa State University Meat Laboratory, nine 2.54-cm thick boneless loin chops were removed from each loin at 1 d postmortem, external adipose tissue was trimmed, and each chop was weighed. Three chops per animal were stored in a sealed plastic bag under atmospheric pressure at 4°C (Lonergan et al, 2001) for an additional 24 h, 48 h, and 96 h for drip loss evaluation (nine chops total). The chops were removed from each bag and weighed after 24 h, 48 h and 96 h storage (48 h, 72 h and 120 h postmortem). Drip loss was recorded as a percentage of weight lost during storage. Chops were then vacuum packaged and stored at -20°C until further biochemical analysis.

Whole Muscle Sample Preparation. Ten pigs from each group (line 2/high pH, line 2/low pH, line 3/high pH, and line 3/low pH) were selected for western blotting, thus whole muscle samples were made for forty animals per postmortem time point (1 d, 48 h, 72 h, and 120 h). Whole muscle samples were prepared according to the methods of Huff-Lonergan et al. (1996) from previously frozen 1 d, 48 h, 72 h and 120 h postmortem samples. In order to prepare whole muscle samples, a frozen portion (0.2 g) was removed from the center of the longissimus, finely chopped, added to 5 mL whole muscle protein extraction buffer (2% SDS, 10 mM sodium phosphate, pH 7.0), and homogenized. The samples were clarified by centrifugation (1500 x g) at 20°C for 20 min. The protein concentration of the sample was determined using the methods described by Lowry, Rosebrough, Farr and Randall (1951) using prepared reagents (Bio-Rad Laboratories, Hercules, CA). The samples were diluted to a concentration of 6.4 mg/mL using deionized water. One volume of each diluted sample was immediately mixed with 0.5 volume sample/buffer tracking dye solution (3 mM EDTA, 3% SDS, [wt/vol], 30% glycerol [vol/vol], 0.001% pyronin-Y [wt/vol], and 30 mM Tris-HCl, pH 8.0) (Wang, 1982) and 0.1 volume of β-mercaptoethanol for a final sample concentration of 4 mg/mL. Samples were then heated at 50°C for 20 min and stored at -80°C until further analysis of μ-calpain, sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA2), ryanodine receptor (RyR1), and vinculin using western blotting.

SDS-PAGE Electrophoresis Gels. Whole muscle samples were run on polyacrylamide gels (acrylamide: N, N'-bis-methylene acrylamide = 100:1 [wt/wt], 0.1% SDS [wt/vol], 0.05% N,N,N'N-tetramethylethylenediamine (TEMED) [vol/vol], 0.05% ammonium persulfate [wt/vol], and 0.375 M Tris-HCl, pH 8.8) as described by Lonergan et al. (2001). A 9% acrylamide separating gel was used for μ-calpain analysis, an 8%

acrylamide separating gel was used for SERCA2 analysis, a 10% acrylamide separating gel was used for vinculin analysis, and a 6% acrylamide separating gel was used for RYR1 analysis. A 5% acrylamide stacking gel (acrylamide: N, N'-bis-methylene acrylamide = 100:1 [wt/wt], 0.1% SDS [wt/vol], 0.125% N,N,N'N-tetramethylethylenediamine (TEMED) [vol/vol], 0.075% ammonium persulfate [wt/vol], and 0.125 M Tris-HCl, pH 6.8) was used on the 8%, 9% and 10% acrylamide separating gels. A 4% acrylamide stacking gel of the same composition was used for the 6% acrylamide gels.

SDS-PAGE of μ-calpain and RYR1 was done using Hoefer SE280 (10 cm wide x 12 cm tall x 1.5 cm thick) Tall Mighty Small gel units (Hoefer Scientific Instruments, San Francisco, CA). For SDS-PAGE of SERCA2 and vinculin, Hoefer SE260 (10 cm wide x 10 cm tall x 1.5 cm thick) Mighty Small gel units were used (Hoefer Scientific Instruments, San Francisco, CA). For SDS PAGE of μ-calpain, 120 μg of whole muscle sample was loaded per lane and gels were run at 120 V at room temperature with a molecular weight standard (Kaleidoscope Prestained Standard, Cat # 161-0324; BioRad, Richmond, CA). For analysis of RYR1, 160 μg of whole muscle sample was loaded per lane and the gel was run at room temperature overnight at a constant amperage of 3 mA. For SDS-PAGE of SERCA2, 60 μg of whole muscle sample was loaded per lane and was run at a constant voltage of 30 V overnight at room temperature. For SDS-PAGE analysis of vinculin, 80 μg of whole muscle sample was loaded per lane and was run at 30 V overnight at room temperature. An at-death standard (whole muscle sample made from porcine longissimus dorsi immediately post-exsanguination) was loaded on all gels in all systems, and an additional standard for degradation of vinculin (whole muscle sample from porcine longissimus dorsi at 120 h postmortem exhibiting large degree of degradation) was loaded on all gels analyzed for

vinculin. The running buffer used in all systems contained 25 mM Tris, 192 mM glycine, 2 mM EDTA, and 0.1% (wt/vol) SDS.

Transfer Conditions. After electrophoresis, all gels were blotted onto Poly Screen polyvinylidene difluoride (PVDF) transfer membrane (NEN Life Science Products, Inc., Boston, MA). For μ-calpain, SERCA2 and vinculin, a Hoefer TE22 Mighty Small Transphor unit was used to transfer proteins to the PVDF membrane. A constant voltage setting of 90 V for 90 min and a transfer buffer containing 25 mM Tris, 192 mM glycine, and 15% methanol [vol/vol] were used. A refrigerated Lauda RE106 circulating water bath (Brinkman Instruments Inc, Westbury, NY) set at 0.4˚C was used to maintain the temperature of the transfer buffer at refrigerated temperatures (for μ-calpain, SERCA2, and vinculin). Gels for RyR1 were transferred to PVDF membranes using a TE62 Transphor Cooling Unit (Hoefer Scientific Instruments, San Francisco, CA) at a constant amperage of 400 mA for 2 h followed by a constant amperage of 1000 mA for 3 h. The transfer buffer used for this system contained 25 mM Tris, 192 mM glycine, and 10% methanol [vol/vol]. The transfer buffer was kept cold using a Lauda RE106 circulating water bath set at -5.0˚C for 2.5 h followed by -10˚C for an additional 2.5 h.

Western Blotting. Following transfer, PVDF membranes were incubated in 20 mL of a blocking solution composed of PBS-Tween (80 mM disodium hydrogen orthophosphate, anhydrous, 20 mM sodium dihihydrogen orthophosphate, 100 mM sodium chloride, 0.1% polyoxyethylene sorbitan monolaurate (Tween-20) [vol/vol]) and 5% non-fat dry milk [wt/vol] for 1 h at room temperature. After blocking, membranes were placed in a primary antibody specific to the protein desired. Primary antibodies used in the western blotting procedure included monoclonal (mouse) anti-μ-calpain (Cat # MA3-940; Affinity

BioReagents, Golden, CO) incubated for a minimum of 12 h (4°C) at a 1:10,000 dilution in PBS-Tween; monoclonal(mouse) anti-SERCA-2 ATPase (Cat # MA3-919; Affinity BioReagents, Golden, CO) for 2 h (room temperature) at a dilution of 1:20,000 in PBS-Tween; monoclonal (mouse) anti RyR1 (Cat # MA3-925; Affinity BioReagents, Golden, CO) for a minimum of 12 h (4°C) at a 1:40,000 dilution in PBS-Tween; and monoclonal mouse anti-human vinculin (Cat # V9131; Sigma-Aldrich, Inc, St. Louis, MO) for 2 h (room temperature) at a dilution of 1:10,000 with PBS-Tween. Following primary antibody incubation, blots were washed three times, 10 min per wash, in PBS-Tween at room temperature. Incubation with secondary antibody followed. Secondary antibody for all proteins was sheep-anti-mouse horseradish peroxidase (HRP) (Cat # NA931; Amersham Biosciences, Piscataway, NJ) at a 1:5000 dilution in PBS-Tween. Blots were washed in PBS-Tween three times, 10 min per wash, prior to detection. A chemiluminescent system was used to detect the labeled protein bands. The detection reagents used were ECL Plus kit (Amersham Pharmacia Biotech, Piscataway, NJ) for μ-calpain, SERCA2, and vinculin while ECL-Advance kit (Amersham Pharmacia Biotech, Piscataway, NJ) was used for RYR1. Both kits were used according to manufacturer's instructions. A 16 bit megapixel charge coupled device (CCD) camera FluorChem 8800 (Alpha Innotech Corporation, San Leandro, CA) and FlourChem IS-8800 software (Alpha Innotech, San Leandro, CA) were used to detect chemiluminescence. Densitometric measurements were determined using AlphaEase FC Version 3.1.2 (Alpha Innotech, San Leandro, CA). For analysis of μ-calpain, the 80 kDa subunit was measured using densitometry while for SERCA2 the intact band was evaluated. Densitometric measurements for RyR1 were determined on the intact band as well as two degradation products termed in this study as band 2 and the doublet band. For analysis of

vinculin, intact metavinculin and vinculin were measured as well as two degradation products considered bands D2 and D3. The final values for the 80 kDa subunit of μ-calpain, intact SERCA, intact RyR1, intact vinculin, intact metavinculin as well as the RyR1 degradation products (band 2 and doublet) were determined as a ratio of protein in the sample compared to an at-death standard. The final values for vinculin degradation products (D2 and D3) were determined as a ratio of protein in the sample compared to a degradation standard.

Statistical Analysis. Data were analyzed using the general linear models procedure of SAS (version 8.2, SAS Institute, Cary, NC). Means were separated using Tukey's test and least squares means were computed. Each of the pigs selected within group served as replicates for pH (45 min, 3 h, 6 h, and 24 h postmortem), drip loss analysis (24 h, 48 h, and 96 h storage), and analysis of μ-calpain, RYR1, SERCA2, and vinculin. Significance was determined at the $P < 0.05$ level. Main effects were separated using PDIFF when least squares means were determined significant ($P < 0.05$) in the analysis of variance table. Data was additionally analyzed using the PROC CORR procedure of SAS. Significance of the correlation was determined at the $P < 0.05$ level.

Results and Discussion

pH and drip loss data. The rate and extent of pH decline has long been known to influence meat color and water-holding capacity (Briskey, 1964). In the current study, no significant differences were observed between lines at 45 min postmortem for the initial 309 pigs measured. The average pH for both lines at this time was 6.00 ± 0.02. At 3 h

postmortem, the average pH (6.00 ± 0.02) measured in line 2 was significantly higher ($P < 0.01$) than the average pH (5.86 ± 0.02) in line 3.

Pigs were selected according to high and low pH measurements at 3 h postmortem in order to obtain groups with different early postmortem pH measurements; thus, as expected, there were significant differences ($P < 0.01$) within lines between high and low pH measurements at 3 h postmortem for the 40 pigs selected for more detailed evaluation (Table 2). Additionally, the high and low pH groups were also significantly different ($P < 0.05$) at 45 min and 6 h (Table 2). At 24 h postmortem, the average pH (5.86) of the longissimus muscles from line 2/high pH group was significantly higher ($P < 0.05$) than all other groups (Table 2).

Drip loss in the longissimus dorsi was affected by pH. The current study showed that the line 2/high pH group had the least amount of drip loss ($P < 0.01$) after 24 h of storage in comparison to line 2/low and line 3/low pH groups, but was not different than line 3/high pH group (Table 2). After 48 h of storage, the high and low pH groups were significantly different ($P < 0.05$) within both lines (Table 2). Additionally, the line 2/high pH group had significantly less ($P < 0.05$) drip loss at 48 h storage than the line 3/low pH group but was not different from the line 3/high group (Table 2). After 96 h of storage, the line 2/high pH group exhibited the least amount of drip loss of all groups ($P < 0.05$; Table 2).

A significant ($P < 0.01$) negative correlation existed between pH at 45 min, 3 h, 6 h, and 24 h postmortem and drip loss at 24 h, 48 h, and 96 h of storage (Table 3). This indicates that the lower pH values at all measured time points are highly related to lower water-holding capacity of pork LD muscle.

The observations between drip loss and pH in this study are in agreement with Warriss and Brown (1987) who found significant negative correlations between 45 min pH and drip loss at 48 h. Similarly, van Laack et al. (1994) observed significant negative correlations between 45 min pH and drip loss at 72 h. Bowker, Wynveen, Grant and Gerrard (1999) observed a similar relationship between pH and drip loss in pork carcasses. The authors saw that the loins from electrically stimulated pork carcasses (lower early postmortem pH) had a greater percent drip loss ($P < 0.05$) than the carcasses that had not been treated with electrical stimulation. This coincides with our results that carcasses with a lower early postmortem pH and/or a lower ultimate pH also had a higher amount of drip loss.

Other studies have shown relationships between lower early postmortem pH and/or a lower ultimate pH and increased drip loss, which coincide with our results. Joo, Kauffman, Kim and Park (1999) showed that animals with a lower ultimate pH had increased drip loss. Lonergan et al. (2001) found that pigs selected for lean growth efficiency, but that were free of the halothane and RN^- gene, had a significantly faster rate of pH decline than the control line. This study also found that the animals with the faster rate of pH decline also had a greater amount of drip loss in the longissimus dorsi, semimembranous, and semitendinous (Lonergan et al., 2001).

As a low early postmortem pH may indicate an increased rate of pH decline, the relationship between low early pH and increased drip loss may be attributed to higher rate of protein denaturation. Offer and Knight (1988) showed that a faster rate of pH decline while the carcass temperature was still warm could result in more protein denaturation. This can be detrimental to water-holding capacity as contractile proteins such as myosin may undergo extreme denaturation, which would alter the crossbridges between actin and myosin and

cause the distance between the two proteins to shrink (van Laack, 1999). This lateral shrinkage would subsequently result in more myofibrillar water to be expelled into the extracellular spaces and thus decrease water-holding capacity.

Color Analysis. Results of the Minolta color analysis at 24 h postmortem revealed differences in the LD muscles from pigs evaluated in this study. At 24 h postmortem, the LD muscles from the line 3/low pH group had a higher ($P < 0.05$) mean reflectance value (L* = 49.49) than the other 3 groups (Table 2). No significant differences between groups were observed for a* or b* values (Table 2). The samples from line 3/low pH group also had a lower pH at 45 min, 3 h, and 6 h postmortem than the line 3/high pH group and were different from the line 2/high pH group at all measured time points. This data suggests that LD from pigs that had a lower early postmortem pH also had a lighter fresh pork color.

Our findings are in agreement with Enfält et al. (1993) who found that pigs exhibiting a paler meat color also had a greater amount of drip. Claeys, De Smet, Demeyer, Geers and Buys (2001) also found that pigs with a faster rate of pH decline had a higher CIE L* value ($P < 0.05$). It is possible that protein denaturation that occurs as a result of a faster rate of pH decline may be likely to induce a higher L* value (Rees, Trout and Warner, 2003). Alternatively, the lighter appearance (higher L* value) may be due to the acceleration of post slaughter metabolism combined with a lower muscle pH at rigor (Rees et al., 2003). The lower pH may cause an increase in free water at the cut surface of the cell, which would result in an increased reflectance and subsequently paler colored meat (Pearson and Dutson, 1985).

Autolysis of μ-calpain. The ubiquitous enzyme μ-calpain has the ability to undergo autolysis, or self proteolytic activity, when in the presence of Ca^{2+} (Suzuki et al., 1981a;

Suzuki, Tusji, Kubata, Kimura and Imajori, 1981b; Hathaway, Werth and Haeberle, 1982). Autolysis results in the removal of a 14 amino acid sequence from the N-terminus of the 80 kDa subunit which forms an active 78 kDa intermediate product (Johnson and Guttman, 1997). An additional 12 amino acids are then removed from the N-terminal end of the 78 kDa subunit to form a 76 kDa subunit in the active state (Johnson and Guttman, 1997; Zimmerman and Schlaepfer, 1991). Autolysis of the small 30 kDa regulatory subunit results in the loss of 91 amino acids from the N-terminus (the Gly-rich region) to form an 18 kDa subunit in both μ- and m-calpain (Goll, Thompson, Li, Wei and Cong, 2003). It is important to note that more Ca^{2+} is required to initiate autolysis than to initiate activity of unautolyzed calpain (Cong, Goll, Peterson, and Kapprell, 1989). Autolysis lowers the Ca^{2+} required for activity, but the exact manner in which this occurs is unknown (Suzuki et al., 1981a; Cottin, Thomson, Sathe, Szpacenko and Goll, 2001). As the physiological Ca^{2+} concentration is lower than is required for μ-calpain to be activated (Suzuki et al., 1981a), autolysis can play an important role in the activation of μ-calpain. With increasing autolysis, μ-calpain is more likely to be proteolytically active at lower levels of Ca^{2+}.

In our study, differences in the level of autolysis were observed at 1 d postmortem in the LD (Table 4). More autolysis, suggested by the loss of the 80 kDa subunit, occurred in the line 2/high pH group than the line 2/low pH group at 1 d postmortem ($P < 0.05$; Figure 1). Additionally, more autolysis was observed in the line 3/high pH group than the line 2/low pH group at 1 d postmortem ($P < 0.05$; Figure 1). This suggests that samples with a higher early postmortem pH had increased autolysis at 1 d postmortem. No differences were found at 48 h postmortem in the samples.

Increased autolysis of calpain was associated with lower pH (Table 2; Table 4). The presence of the intact 80 kDa subunit of μ-calpain at 1 d postmortem was negatively correlated to pH at 3 h ($P < 0.01$) and 6 h ($P < 0.05$) postmortem (Table 5). At 48 h postmortem, degradation of the 80 kDa subunit was negatively correlated with 45 min ($P < 0.05$), 3 h ($P < 0.01$), 6 h ($P < 0.05$), and 24 h ($P < 0.05$) pH values (Table 5). These correlations suggest that longissimus muscles with higher pH have increased autolysis of the 80 kDa subunit of μ-calpain at 1 d and 48 h postmortem.

Our findings concerning the relationship between high pH and increased μ-calpain autolysis have been shown by several other authors. The optimum pH for calpain activity is 7.4 - 7.6 (Edmunds, Nagainis, Sathe, Thomson and Goll, 1991). However, postmortem conditions of meat are at a pH of approximately 5.5. Thus, it would be expected that the calpains would less active or undergo autolysis more slowly with the lower pH conditions found in postmortem meat. This is supported by our study as we found that samples with a higher early postmortem pH had increased autolysis. Koohmaraie, Schollmeyer and Dutson (1986) showed that in conditions similar to postmortem aging with a pH 5.5 to 5.8 and at 5°C, μ-calpain retained 24-28% of its maximum activity at pH 7.5 and at 25°C, which suggests that a sample with a higher pH would have greater calpain activity. Claeys et al. (2001) supported these findings when they observed a lower μ-calpain activity in the genetic line with a faster rate of pH decline than the genetic line with a slower rate of pH decline.

Proteolysis of myofibrillar proteins by the calpains can affect water-holding capacity. In the current study, μ-calpain autolysis was also related to drip loss. The presence of the 80 kDa subunit of μ-calpain at both 1 d and 48 h postmortem was positively correlated ($P < 0.05$) to drip loss after 24 h, 48 h and 96 h of storage (Table 5). These correlations suggest

that samples that exhibited less intact 80 kDa μ-calpain also exhibited a lower percent of drip loss. This could indicate that increased autolysis may be occurring, and as autolysis has been shown to parallel activation (Baki, Tompa, Alexa, Molnar and Fredrich, 1996), increased substrate proteolysis by calpain may also result, particularly of those proteins involved in water-holding capacity.

Ryanodine Receptor (RyR1) analysis. The ryanodine receptor (RyR1) is a 565 kDa protein found in the lateral cisternae of the sarcoplasmic reticulum in skeletal muscle that is responsible for Ca^{2+} release into the sarcoplasm (Froemming, Murray, Harmon, Pette and Ohlendieck, 2000; Sutko et al., 1991). In skeletal muscle, release of Ca^{2+} by the RyR1 contributes to muscle contraction. The calpains and calpastatin have been found to be associated with cellular membranes and further localization has uncovered that calpain activity is associated with the transverse tubule membranes (Brandt et al., 1992), a site which is involved in a protein-protein interaction with the RyR1 (Proenza et al., 2002).

Our analysis revealed no significant differences in degradation of the intact band of RyR1 between or within the four groups (Table 4). However, significant differences ($P < 0.05$) as determined by densitometry were noted at 48 h postmortem between the line 2/low pH group and the line 3/high pH group for degradation of RyR1 immunoreactive bands that migrated as a doublet (Table 4; Figure 2). The doublet was comprised of two lower molecular weight bands that were immunoreactive with the monoclonal antibody specific for RyR1 and that appeared in samples with increased proteolysis of the intact RyR1 band, thus the doublet is most likely a degradation product of this protein. This RyR1 degradation product was more prominent in the line 3/high pH group than the line 2/low pH group at 48 h postmortem. Additionally, less intensity of a higher migrating degradation product (band 2)

was observed in line 2 than line 3 at 48 h postmortem (Table 4; Figure 3). Band 2 was considered an intermediate degradation product in this study as it was also immunoreactive with the RyR1 monoclonal antibody and the intensity of this band decreased or disappeared at later times postmortem as lower migrating bands were increasingly detected. When evaluating band 2 in our study, it was observed that there was more degradation in line 3 than line 2 at 48 h postmortem ($P < 0.01$).

Degradation of the RyR1 (band 2) and pH decline were found to be related. The intermediate degradation product (band 2) of RyR1 was negatively correlated to pH at 6 h ($P < 0.05$) and 24 h ($P < 0.01$) postmortem (Table 5). This indicates that increased proteolysis of RyR1 is associated with lower pH. Additionally, degradation of RyR1 (band 2) was correlated to drip loss in this study. Band 2 of RyR1 at 48 h PM was strongly positively correlated ($P < 0.01$) with drip loss at 24 h, 48 h, and 96 h storage. This indicates that samples with a higher amount of degradation of RyR1 also had decreased water-holding capacity as indicated by increased drip loss. Thus, it can be suggested by our data that increased proteolysis of RyR1 was found in pork LD samples that had a lower pH, which subsequently may have caused poorer water-holding capacity.

The RyR1 is a substrate of μ-calpain (Gilchrist, Wang, Katz and Belcastro, 1992; Iino et al., 1992; Shoshan-Barmatz et al., 1994). Gilchrist et al. (1992) found that when RyR1 was exposed to μ- and m-calpain, a 410 kDa and 150 kDa degradation product was produced. In our study, autolysis of the 80 kDa subunit of μ-calpain at 1 d postmortem was positively correlated with degradation of the intact RyR1 at 1 d and 48 h (Table 6). Autolysis of the 80 kDa μ-calpain subunit at 48 h postmortem was also positively correlated with proteolysis of intact RyR1 at 1 d and 48 h (Table 6). The 80 kDa subunit at 1 d and 48 h was negatively

correlated to degradation of the RyR1 doublet at 48 h postmortem (Table 6). These correlations suggest that in samples with more degradation of RyR1 also had undergone a greater degree of autolysis of the 80 kDa subunit.

The current study observed relationships between increased μ-calpain autolysis and increased degradation of RyR1. Additionally, we found that increased RyR1 proteolysis and increased drip loss were positively correlated. It can be hypothesized that proteolysis of the RyR1 by calpain may promote the release of Ca^{2+} during the postmortem time period, which could cause the level of sarcoplasmic Ca^{2+} to rise. The increased concentration of Ca^{2+} may promote the further activation of μ-calpain which may result in degradation of proteins that affect water-holding capacity.

Our hypothesis can be supported by the work of other authors. Shoshan-Barmatz et al. (1994) showed that cleavage of the RyR1 by calpain stimulated Ca^{2+} release. Thus, this degradation could allow Ca^{2+} leakage which may elevate the Ca^{2+} ion concentration enough to further activate the calpains. Iino et al. (1992) reported that the RyR1 released Ca^{2+} at an increased maximum rate when degraded by m-calpain. It is possible that degradation of the RyR1 by calpain may alter the channel's ability to function and could stimulate a large influx of Ca^{2+} into the cell sarcoplasm (Iino et al., 1992). This loss of function could be detrimental to pork quality by causing an increased metabolism and thus an accelerated rate of pH decline and/or a lower ultimate pH, which could result in poor water-holding capacity. However, a slight alteration to the RyR1 and its function could result in a sufficient localized sarcoplasmic Ca^{2+} increase that may activate μ-calpain, and possibly m-calpain, which could subsequently promote the further degradation of RyR1 and other muscle proteins associated

with water-holding capacity. More research is needed to explore the relationship between degradation of the skeletal muscle RyR1 and the calpain system.

Sarcoplasmic Reticulum Ca^{2+}-ATPase analysis. The sarcoplasmic reticulum Ca^{2+}-ATPase pump (SERCA) is involved in calcium regulation by removing calcium from the sarcoplasm and returning it to the sarcoplasmic reticulum. This 110 kDa transmembrane protein is ATP-dependent and exists as several different isoforms. SERCA1 and SERCA2 are expressed in adult skeletal muscle. No visual alterations were observed in preliminary data evaluating SERCA1 in the samples used for this study, however, changes in band density were observed for SERCA2. Thus, this isoform was evaluated.

This study found no significant differences in degradation of intact SERCA2 between or within groups. However, degradation of intact SERCA2 was related to pH. The presence of intact SERCA2 at 1 d and 72 h postmortem was positively correlated ($P<0.05$) with 24 h pH measurements (Table 5). These correlations suggest in products with a high early postmortem and/or high ultimate pH, there is more intact SERCA2.

Correlations also exist between intact SERCA2 and drip loss. A negative correlation ($P < 0.05$) is shown between intact SERCA at 120 h postmortem and drip loss at 24 h storage (Table 5). Therefore, less intact SERCA2 is associated with increased drip loss after 24 h of storage.

A decrease in activity of the SERCA pump could be detrimental to pork quality early postmortem by limiting the amount of Ca^{2+} that could be removed from the sarcoplasm back to the sarcoplasmic reticulum. The subsequent higher concentration of Ca^{2+} in the sarcoplasm could promote increased muscle contraction and a faster rate of anaerobic metabolism which could ultimately lead to a faster rate of pH decline. This is in agreement

with our study as we observed that increased degradation of intact SERCA2 was closely associated with decreased pH. It may be suggested that degradation of the pump is causing the Ca^{2+} concentration to rise intracellularly which would result in a faster rate of muscle metabolism and acidic conditions. Additionally, the current study also showed that increased degradation of SERCA2 was related to more drip loss after 24 h of storage.

In this study, no correlation was shown between the presence of the 80 kDa μ-calpain subunit at 24 and 48 h and degradation of SERCA2. However, Asmus, Rowe, Berg, Lonergan and Huff-Lonergan (2003) showed that an increase in degradation of SERCA1 was related to an increase in the extent of μ-calpain autolysis.

Vinculin. At 1 d, 48 h, 72 h and 120 h, western blotting of vinculin revealed no significant differences in degradation of intact metavinculin, intact vinculin or band D3 (furthest migrating degradation product of vinculin in our study) between the four groups of pigs (Table 7). In samples taken at 48 h postmortem, western blotting revealed increased ($P < 0.05$) degradation of vinculin (more D2 degradation product of vinculin) for the line 3/high pH and the line 3/low pH group than the line 2/low pH group (Figure 4).

Correlations were also observed between vinculin and pH. Degradation of vinculin (D3 band) at 48 h was positively correlated ($P < 0.05$) to pH measured at 45 min postmortem (Table 5). These results suggest that products with a higher early postmortem pH had increased degradation of the costameric protein vinculin. As we showed earlier that μ-calpain had undergone more autolysis at higher pH conditions, and autolysis parallels activity (Baki et al., 1996), it may be suggested that μ-calpain is more active. Thus, proteolysis of calpain substrates such as vinculin would also be likely to occur with increasing pH. This can be further supported as autolysis of μ-calpain and degradation of vinculin were correlated

in this study. Presence of the 80 kDa subunit of μ-calpain at 1 d postmortem was negatively correlated ($P < 0.05$) to presence of a vinculin degradation product at 48 h (Table 8). Presence of the 80 kDa subunit of μ-calpain at 48 h postmortem was also negatively correlated ($P < 0.05$) to the presence of a degradation product of vinculin at 48 h (Table 8). This indicates that samples that had a greater degree of autolysis of the 80 kDa subunit at 24 h postmortem also had more degradation of vinculin. It is noted that a positive correlation ($P < 0.01$) was observed between μ-calpain at 48 h PM and vinculin (band D3) at 72 h PM. It may be hypothesized that μ-calpain is less autolyzed at 48 h PM, which would allow for more potential for further degradation of vinculin at later times postmortem. Thus, this could explain the positive correlation observed between 48 h μ-calpain and 72 h vinculin and would coincide with the relationships between μ-calpain at 1 d and 48 h PM and vinculin at 48 h PM.

Degradation of the costameric protein vinculin was related to degradation of intact RyR1. Presence of intact RyR1 at 48 h postmortem was negatively correlated with a vinculin degradation product at the same time postmortem ($r = -0.64$; $P < 0.01$) as well as both proteins negatively correlating at 120 h postmortem ($r = -0.32$; $P < 0.01$). This indicates that in samples with increased degradation of intact RyR1, a greater degree of proteolysis of vinculin was also observed.

Interestingly to note, correlations also existed between degradation of SERCA2 and vinculin. A negative correlation ($r = -0.3133$; $P < 0.05$) was shown between intact SERCA2 and a vinculin degradation product at 72 h. Our results indicate that products with more extensive SERCA2 degradation had increased proteolysis of vinculin as expressed by

increased area of a degradation product. This relationship was similar to that observed between RyR1 and vinculin.

Conclusions

This study observed relationships between pH decline, μ-calpain autolysis, proteolysis of the costameric protein vinculin, and degradation of membrane proteins involved in calcium homeostasis. In this study, pigs with a lower early postmortem and/or ultimate pH were shown to have increased proteolytic cleavage of proteins involved in Ca^{2+} regulation. Increased μ-calpain autolysis was correlated to degradation of RyR1, which could indicate a loss of function of this Ca^{2+} regulating protein. The loss of function could promote further degradation by calpain due to the increase of Ca^{2+} in the sarcoplasm. Additionally, degradation of other myofibrillar and cytoskeletal proteins such as vinculin may be initiated. Degradation of RyR1 was shown to occur more rapidly in samples with a lower early postmortem and/or ultimate pH, which could result in poorer water-holding capacity. Samples that had increased autolysis of μ-calpain were shown to have more proteolysis of intact RyR1. Increased degradation of SERCA2 was correlated to lower early postmortem and/or ultimate pH and more drip loss, which suggests that degradation of this calpain substrate may destabilize calcium regulation and negatively affect pork quality. In this study, animals with a higher early postmortem pH and improved water-holding capacity also had increased μ-calpain autolysis and vinculin degradation. These results may aid in directing additional research in uncovering the mechanisms that drive variations in pH decline and water-holding capacity. Further studies are needed to fully understand the

relationship between Ca^{2+}-regulating proteins and μ-calpain in order to understand how their interactions affect pork quality.

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Table 1. Means, minimums, and standard deviations of variables used in Pearson correlation analysis of the longissimus dorsi (LD).

Variable	N	Mean	Min	Max	Std Deviation
pH 45 min	40	6.10	6.50	5.62	0.20
pH 3 h	40	5.83	5.27	6.43	0.41
pH 6 h	40	5.65	5.32	6.10	0.22
pH 24 h	40	5.68	5.41	6.41	0.17
% Drip Loss 24 h	40	2.36	0.00	5.42	1.32
% Drip Loss 48 h	40	2.60	0.52	6.29	1.43
% Drip Loss 96 h	40	3.83	0.00	7.14	1.85
SERCA2 Intact 1 d[1]	40	38.00	7.03	86.78	18.30
SERCA2 Intact 48 h[1]	40	33.45	5.02	119.52	30.10
SERCA2 Intact 72 h[1]	40	30.24	4.42	73.49	19.51
SERCA2 Intact 120 h[1]	40	30.99	5.04	102.09	23.73
RyR1 Intact 1 d[1]	40	61.72	0.00	139.80	35.47
RyR1 Intact 48 h[1]	40	41.26	0.00	102.12	32.78
RyR1 Intact 72 h[1]	40	18.90	0.00	100.56	26.84
RyR1 Intact 120 h[1]	40	3.11	0.00	74.32	12.53
RyR1 Band 2 48 h	40	20.66	0.00	73.81	21.83
RyR1 Doublet 48 h	40	90.19	0.00	182.48	54.11
μ-calpain 80 kDa 1 d[1]	40	8.16	0.00	80.63	19.16
μ-calpain 80 kDa 48 h[1]	40	5.53	0.00	52.29	12.59
Vinculin Deg(3) 1 d[1]	40	14.68	0.00	109.04	20.72
Vinculin Deg(3) 48 h[1]	40	20.16	0.00	71.68	22.92
Vinculin Deg(3) 72 h[1]	40	40.80	0.00	187.8	41.36
Vinculin Deg (3) 120 h[1]	40	66.42	0.00	155.50	38.61

[1] Value expressed as % of standard

Table 2. Least squares means and standard errors of longissimus muscle pH measurements during the first 24 h postmortem, L*, a* and b* values at 24 h postmortem, and pork loin chop drip loss measurements during 24, 48 and 96 h of storage for pigs (n = 40) selected based on rate of pH decline.

	Line 2/High[1]	Line 2/Low[2]	Line 3/High[3]	Line 3/Low[4]	SEM
pH					
45 min PM	6.21^{y}	5.91^{z}	6.27^{y}	6.02^{z}	0.046
3 h PM	6.27^{a}	5.47^{b}	6.19^{a}	5.41^{b}	0.029
6 h PM	5.86^{a}	5.49^{b}	5.78^{a}	5.47^{b}	0.046
24 h PM	5.86^{a}	5.65^{b}	5.66^{b}	5.54^{b}	0.042
Minolta					
L*	41.46^{b}	44.32^{b}	44.67^{b}	49.49^{a}	0.984
a*	0.0815^{a}	0.7720^{a}	-0.3111^{a}	0.7514^{a}	0.359
b*	8.889^{a}	9.730^{a}	9.157^{a}	10.524^{a}	0.446
% Drip Loss					
24 h storage	1.31^{b}	2.98^{a}	$1.92^{a,b}$	3.23^{a}	0.350
48 h storage	1.20^{y}	$2.89^{x,z}$	$2.39^{y,z}$	3.91^{x}	0.339
96 h storage	1.92^{z}	4.37^{y}	3.96^{y}	5.06^{y}	0.467

[1] Longissimus dorsi from line 2 pigs with a high pH at 3 hrs postmortem; n = 10.
[2] Longissimus dorsi from line 2 pigs with a low pH at 3 hrs postmortem; n = 10.
[3] Longissimus dorsi from line 3 pigs with a high pH at 3 hrs postmortem; n = 10.
[4] Longissimus dorsi from line 3 pigs with a low pH at 3 hrs postmortem; n = 10.
^{a,b,c,d} Means within rows lacking a common letter differ $P < 0.01$ (SEM).
^{x,y,z} Means within rows lacking a common letter differ $P < 0.05$ (SEM).

Table 3. Pearson correlations between the LD quality attributes for 45 min, 3 h, 6 h, and 24 h postmortem pH and 24 h, 48 h, and 96 h storage drip loss.

	pH 45 min	pH 3 h	pH 6 h	pH 24 h	Drip Loss 24 h	Drip Loss 48 h	Drip Loss 96 h
pH 45 min	x	**0.66***	**0.54***	0.23	**-0.49***	**-0.48***	**-0.52***
pH 3 h		x	**0.82***	**0.55***	**-0.59***	**-0.63***	**-0.55***
pH 6 h			x	**0.65***	**-0.56***	**-0.61***	**-0.62***
pH 24 h				x	**-0.62***	**-0.72***	**-0.75***
Drip Loss 24 h					x	**0.61***	**0.69***
Drip Loss 48 h						x	**0.89***
Drip Loss 96 h							x

* Significant correlation between X and Y at the $P < 0.01$ level.

Table 4. Least squares means and standard errors of μ-calpain 80 kDa subunit, intact RyR1 and intact SERCA2 area expressed as % of At-death porcine standard.

	Line 2/High[1]	Line 2/Low[2]	Line 3/High[3]	Line 3/Low[4]	SEM
μ-calpain 80 kDa					
1 d PM	0.000^{z}	23.014^{y}	0.000^{z}	$9.633^{y,z}$	5.547
48 h PM	0.000^{a}	10.195^{a}	0.000^{a}	11.933^{a}	3.705
RyR1 Intact					
1 d PM	60.263^{a}	62.273^{a}	61.746^{a}	62.598^{a}	11.671
48 h PM	34.974^{a}	54.610^{a}	30.515^{a}	44.955^{a}	10.330
72 h PM	14.669^{a}	7.064^{a}	23.453^{a}	30.417^{a}	8.331
120 h PM	0.908^{a}	0.000^{a}	0.000^{a}	11.536^{a}	3.789
RyR1 Band 2					
48 h PM	8.056^{b}	16.508^{b}	25.535^{a}	32.536^{a}	8.255
RyR1 Doublet					
48 h PM	$86.550^{a,b}$	50.907^{b}	124.971^{a}	$98.319^{a,b}$	15.444
SERCA2 Intact					
1 d PM	45.355^{a}	39.564^{a}	30.397^{a}	36.686^{a}	5.751
48 h PM	38.866^{a}	37.672^{a}	27.841^{a}	29.432^{a}	9.774
72 h PM	34.789^{a}	33.236^{a}	33.052^{a}	19.871^{a}	5.751
120 h PM	38.938^{a}	33.901^{a}	24.619^{a}	26.492^{a}	7.572

[1] Longissimus dorsi from line 2 pigs with a high pH at 3 hrs postmortem; n = 10.
[2] Longissimus dorsi from line 2 pigs with a low pH at 3 hrs postmortem; n = 10.
[3] Longissimsus dorsi from line 3 pigs with a high pH at 3 hrs postmortem; n = 10.
[4] Longissimus dorsi from line 3 pigs with a low pH at 3 hrs postmortem; n = 10.
[a,b] Means within rows lacking a common letter differ $P < 0.01$ (SEM).
[y,z] Means within rows lacking a common letter differ $P < 0.05$ (SEM).

Table 5. Pearson correlations between the LD 45 min, 3 h, 6 h, and 24 h postmortem pH, drip loss at 24 h, 48 h, and 96 h storage; μ-calpain autolysis, intact SERCA2 degradation, intact RyR1 degradation, and vinculin band D3 at 1 d and 48 h postmortem; and RyR1 Band 2 and doublet degradation at 48 h postmortem.

	pH 45 min	pH 3 h	pH 6 h	pH 24 h	Drip Loss 24 h	Drip Loss 48 h	Drip Loss 96 h
μ-calpain 80 kDa 1 d	-0.31	**-0.40[1]**	**-0.38[2]**	-0.29	**0.57[1]**	**0.34[1]**	**0.33[2]**
μ-calpain 80 kDa 48 h	**-0.35[2]**	**-0.44[1]**	**-0.40[2]**	**-0.34[2]**	**0.66[1]**	**0.47[1]**	**0.45[1]**
SERCA2 Intact 1 d	-0.15	0.01	0.10	**0.39[2]**	-0.16	-0.07	-0.17
SERCA2 Intact 72 h	-0.13	0.19	0.02	**0.43[1]**	-0.14	-0.15	-0.12
SERCA2 Intact 120 h	-0.13	0.02	0.15	0.29	**-0.35[2]**	0.05	-0.06
RyR1 Intact 1 d	-0.10	-0.04	-0.06	-0.12	**0.36[2]**	0.23	0.27
RyR1 Intact 48 h	**-0.38[2]**	-0.29	**-0.38[2]**	-0.14	**0.41[1]**	0.31	**0.38[2]**
RyR1 Intact 120 h	-0.19	-0.25	-0.23	-0.18	0.14	**0.32[2]**	0.25
RyR1 Band 2 48 h	-0.16	-0.26	**-0.33[2]**	**-0.44[1]**	**0.51[1]**	**0.51[1]**	**0.54[1]**
RyR1 Doublet 48 h	**0.50[1]**	0.22	0.07	-0.08	**-0.33[2]**	-0.17	-0.22
Vinculin Band D3 48 h	**0.32[2]**	0.21	0.25	0.02	-0.28	-0.04	-0.12

[1] Significant correlation between X and Y at the $P < 0.01$ level.
[2] Significant correlation between X and Y at the $P < 0.05$ level.

Table 6. Pearson correlations between μ-calpain autolysis at 1 d and 48 h, intact RyR1 degradation, RyR1 Band 2 degradation, and RyR1 Doublet degradation at 1 d, 48 h, 72 h, and 120 h postmortem.

	μ-calpain 80 kDa 48 h	**RyR1 Intact 1 d**	**RyR1 Intact 48 h**	**RyR1 Intact 72 h**	**RyR1 Intact 120 h**	**RyR1 Band 2 48 h**	**RyR1 Doublet 48 h**
μ-calpain 80 kDa 1 d	**0.77**[1]	**0.55**[1]	**0.46**[1]	-0.10	-0.01	0.25	**-0.42**[1]
μ-calpain 80 kDa 48 h	x	**0.42**[1]	**0.45**[1]	-0.07	0.03	**0.37**[2]	**-0.42**[1]

[1] Significant correlation between X and Y at the $P < 0.01$ level.
[2] Significant correlation between X and Y at the $P < 0.05$ level.

Table 7. Least squares means and standard errors of Vinculin area expressed as a % of At-death standard (bands AD1, AD2) and Degradation standard (bands D2,D3).

	1 d PM	48 hr PM	72 hr PM	120 hr PM
Metavinculin Intact				
Line 2/High[1]	48.769[z]	23.374[z]	3.937[z]	0.000[z]
Line 2/Low[2]	53.022[z]	84.132[z]	4.516[z]	0.000[z]
Line 3/High[3]	59.959[z]	28.370[z]	10.409[z]	4.466[z]
Line3/Low[4]	95.335[z]	55.578[z]	12.744[z]	68.729[z]
SEM	12.356	19.358	5.987	27.305
Vinculin Intact				
Line 2/High[1]	95.045[z]	97.994[z]	79.571[z]	77.780[z]
Line 2/Low[2]	98.091[z]	79.894[z]	71.821[z]	65.337[z]
Line 3/High[3]	100.522[z]	137.034[z]	67.663[z]	89.126[z]
Line 3/Low[4]	104.685[z]	96.420[z]	80.569[z]	98.810[z]
SEM	8.933	27.450	9.277	17.271
D2				
Line 2/High[1]	68.710[z]	73.543[y,z]	88.255[z]	75.784[z]
Line 2/Low[2]	60.945[z]	36.328[y]	106.274[z]	71.567[z]
Line 3/High[3]	68.213[z]	101.566[z]	94.112[z]	96.206[z]
Line 3/Low[4]	69.611[z]	94.815[z]	125.194[z]	93.301[z]
SEM	10.689	12.257	16.706	8.918
D3				
Line 2/High[1]	14.344[z]	19.240[z]	32.102[z]	67.053[z]
Line 2/Low[2]	12.081[z]	8.898[z]	39.461[z]	61.859[z]
Line 3/High[3]	13.523[z]	29.829[z]	34.144[z]	80.195[z]
Line 3/Low[4]	18.771[z]	22.653[z]	57.493[z]	56.583[z]
SEM	6.768	7.113	13.198	12.367

[1] Longissimus dorsi from PIC280 pigs with a high pH at 3 hrs postmortem; n = 10.
[2] Longissimus dorsi from PIC280 pigs with a low pH at 3 hrs postmortem; n = 10.
[3] Longissimsus dorsi from PIC337 pigs with a high pH at 3 hrs postmortem; n = 10.
[4] Longissimus dorsi from PIC337 pigs with a low pH at 3 hrs postmortem; n = 10.
[y,z] Means within measured bands within columns lacking a common letter differ $P < 0.05$ (SEM).

Table 8. Pearson correlations between μ-calpain autolysis at 1 d and 48 h, intact SERCA2 degradation, and Vinculin Band 2 degradation at 1 d, 48 h, 72 h, and 120 h postmortem.

	SERCA2 Intact Band 1 d	SERCA2 Intact 48 h	SERCA2 Intact 72 h	SERCA2 Intact 120 h	Vinculin Band D3 1 d	Vinculin Band D3 48 h	Vinculin Band D3 72 h	Vinculin Band D3 120 h
μ-calpain 80 kDa 1 d	0.054	-0.03	-0.11	-0.04	-0.26	**-0.38**[2]	0.15	0.06
μ-calpain 80 kDa 48 h	0.02	-0.06	-0.14	-0.09	-0.28	**-0.40**[2]	**0.42**[1]	0.06

[1] Significant correlation between X and Y at the $P < 0.01$ level.
[2] Significant correlation between X and Y at the $P < 0.05$ level.

Figure 1. Western blot of LD samples representing six pigs with different levels of μ-calpain autolysis at 1 d postmortem. Each lane of the 9% SDS-PAGE gels was loaded with 120 μg of protein for each sample. Lane 1 represents an at-death control sample made from porcine longissimus dorsi removed immediately post-exsanguination. Lanes 2 and 3 depict two representative animals from line 3 that had a 3 h pH > 6.0 (line 3/high). Lanes 4 and 5 depict two representative animals from line 2 that had a 3 hr pH > 6.0 (line 2/high). Lanes 6 and 7 depict two representative samples from line 2 that had a 3 h pH < 5.7 (line 2/low).

Figure 1. Western blot of μ-calpain samples 1 day postmortem.

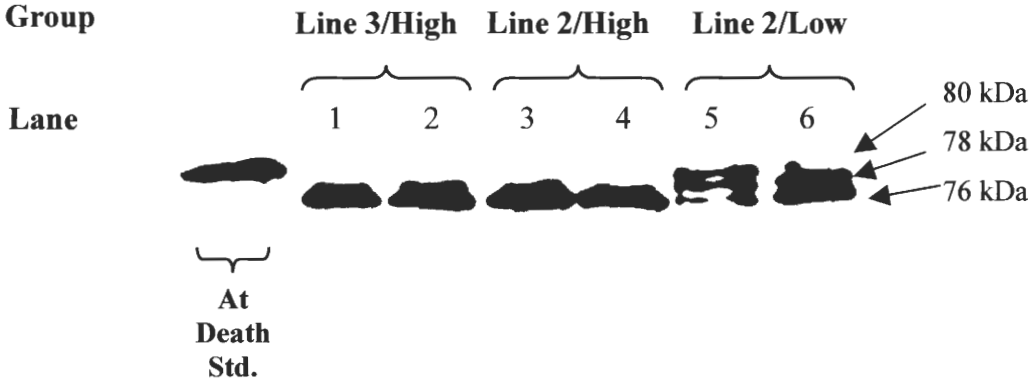


Figure 2. Western blot of LD samples representing two pigs from the line 2/ low pH group and two pigs from the line 3/ high pH groups that had different relative amounts of the RyR1 doublet degradation product at 48 h postmortem. Each lane of the 6% SDS-PAGE gels was loaded with 160 μg of protein for each sample. Lane 1 was loaded with an at-death control sample made from porcine longissimus dorsi removed immediately post-exsanguination. Lanes 2 and 3 depict two representative animals from line 3 that had a 3 h pH > 6.0 (line 3/high). Lanes 4 and 5 depict two representative animals from the line 2 that had a 3 h pH < 5.7 (line 2/low).

Figure 2. Western blot of LD RyR1 degradation (degradation product doublet) at 48 h postmortem.

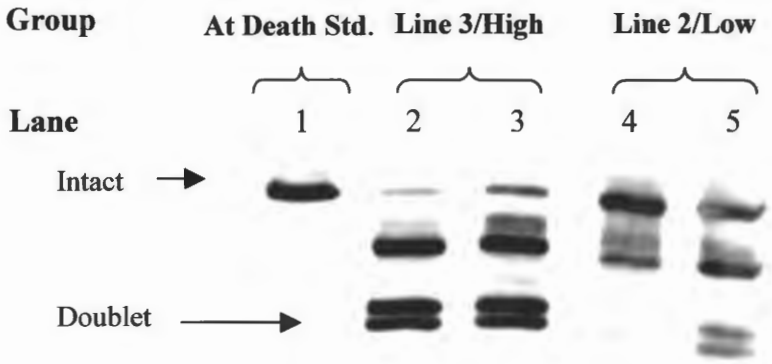


Figure 3. Western blot of LD samples representing two pigs from line 2 and two pigs from line 3 that expressed different relative amounts of the RyR1 Band 2 degradation product at 48 h postmortem. Each lane of the 6% SDS-PAGE gels was loaded with 160 μg of protein for each sample. Lane 1 was loaded with an at-death control sample made from porcine longissimus dorsi removed immediately post-exsanguination. Lanes 2 and 3 depict two representative animals from line 3. Lanes 4 and 5 represent two animals from line 2.

Figure 3. Western blot of LD RyR1 degradation (degradation product Band 2) at 48 h postmortem.

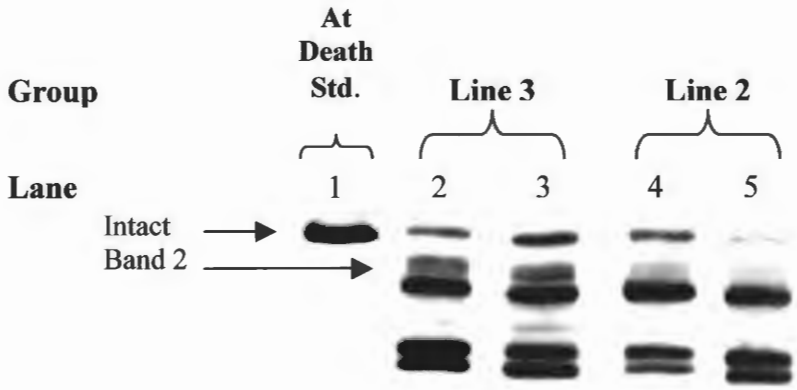
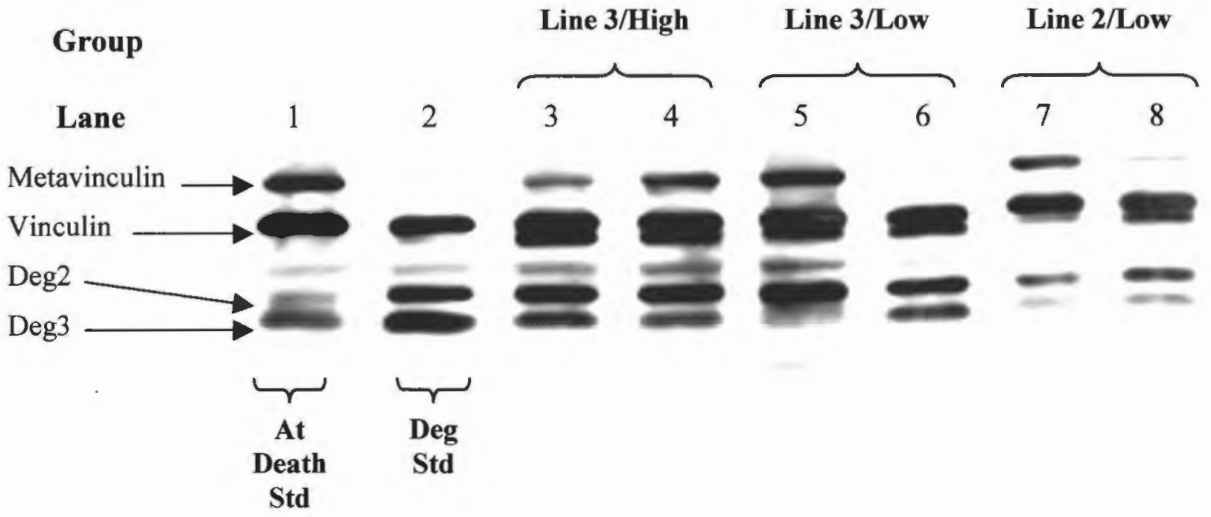


Figure 4. Western blot of LD samples representing six pigs with different vinculin degradation product (D2) at 48 h postmortem. Each lane of the 10% gel was loaded with 80 μg of protein for each sample. Lane 1 represents an at-death control sample made from porcine longissimus dorsi removed immediately post-exsanguination. Lane 2 was loaded with a degradation standard made from porcine longissimus dorsi at 120 h postmortem that exhibited a large degree of degradation. Lanes 3 and 4 depict two representative animals from line 3 that exhibited a 3 h pH > 6.0 (line 3/high). Lanes 5 and 6 represent two animals from line 3 that had a 3 h pH < 5.7 (line 3/low). Lanes 7 and 8 depict two representative animals from line 2 that had a 3 h pH < 5.7 (line 2/low).

Figure 4. Western blot of LD vinculin band D2 degradation at 48 h postmortem.



General Summary

Poor water-holding capacity continues to be an issue in the pork industry. As calcium is an important mediator in muscle metabolism, it is likely that alterations in proteins involved in calcium regulation may be the cause of some unexplained variations in water-holding capacity. The sarcoplasmic reticulum is the major regulator of the Ca^{2+} concentration in skeletal muscle cells, with the ryanodine receptor (RyR1) releasing the sequestered Ca^{2+} into the sarcoplasm and sarcoplasmic reticulum Ca^{2+}-ATPase pumping the released Ca^{2+} back into the sarcoplasmic reticulum. Modification of the RyR1 could cause the channel to remain more fully open while alteration of the SERCA pump could decrease its ability to pump Ca^{2+} out of the cell. These mechanisms could cause an increased metabolism and a decreased pH, which could result in greater drip loss in pork products. Alternatively, an increase in Ca^{2+} concentration could promote the activation of the calpain system, which would subsequently cause the proteolysis of proteins implicated in improved water-holding capacity as well as further degradation of RyR1 and the SERCA pump. The results of this study reveal how proteolytic processes early postmortem involving the calcium-regulating proteins influence pH decline and water-holding capacity.

Quality attributes such as drip loss and color were related to pH in this study. Samples that had a higher 24 h pH displayed less drip loss after 24 and 96 h storage. Our study also found that longissimus muscles from animals with a higher early postmortem and/or ultimate pH tended to have the highest L* values, indicating paler colored meat. It is possible that protein denaturation that occurs as a result of a faster pH decline may be likely

to induce a higher L* value. Alternatively, the lighter appearance may because the lower pH causes an increase in free water at the cell surface, thus the muscle appears paler in color.

Autolysis of μ-calpain was related to pH and drip loss in this study. Samples with higher early postmortem and/or ultimate pH were shown to have increased autolysis of μ-calpain at 1 d postmortem. Additionally, samples that had increased autolysis of μ-calpain also tended to have less drip loss. As autolysis parallels activation, this could suggest that μ-calpain is active and proteolysis of proteins involved in water-holding capacity may be occurring.

Appearance of a specific degradation product of RyR1 was shown to occur more rapidly in animals with a lower early postmortem and/or ultimate pH. This degradation product of RyR1 was found in pork LD samples that had a lower pH, which subsequently may have caused poorer water-holding capacity. It is possible that degradation of the RyR1 by proteolytic enzymes such as the calpains may alter the channel's ability to function and could stimulate a large influx of Ca^{2+} into the cell sarcoplasm. This loss of function could have detrimental effects on pork quality by causing an increased metabolism and thus an accelerated pH decline and/or a lower ultimate pH, which could subsequently result in increased drip loss.

Increased degradation of RyR1 was correlated to samples that had a greater degree of μ-calpain autolysis. It could be hypothesized that the increase of autolysis could have been aided by an increase in sarcoplasmic Ca^{2+} due to increased release by the altered RyR1. The loss of function by the RyR1 could result in an increase of Ca^{2+} that would be sufficient to activate μ-calpain. This increase in μ-calpain activity would likely promote proteolysis of myofibrillar and myofibrillar-associated proteins in addition to Ca^{2+}-regulating proteins.

Increased proteolysis of the intact SERCA2 pump was also found to be related to higher early postmortem and/or ultimate pH in this study. This may suggest that as the Ca^{2+} pump is being degraded, it is unable to function properly; thus the sarcoplasmic Ca^{2+} concentration is rising. This could promote a faster rate of anaerobic metabolism, which would ultimately lead to a lower early postmortem and/or ultimate pH.

The costameric protein vinculin can be degraded by calpain. Proteolysis of vinculin can result in a loss of the structural stability of the muscle fiber, which can be implicated in variations in water-holding capacity. In our study, samples with a higher early postmortem and/or ultimate pH tended to have increased degradation of vinculin. Additionally, samples with more proteolysis of both RyR1 and SERCA2 were shown to have a greater degree of degradation of vinculin. Samples that had increased proteolysis of vinculin also had further autolysis of μ-calpain. Thus, it could be stated that samples with more autolysis of μ-calpain tended to have increased proteolysis of its substrates.

These observations suggest that alterations of the Ca^{2+}-regulation proteins during the postmortem period can affect muscle metabolism and the rate of pH decline. This in turn may affect pork quality by promoting the activation of μ-calpain and, subsequently, the further degradation of the Ca^{2+}-regulation proteins as well as proteolysis of the costameric protein vinculin. This increase in proteolysis could be used as a predictor for water-holding capacity. These results may aid in directing additional research in uncovering the mechanisms that drive variations in pH decline and water-holding capacity. Further studies are needed to fully understand the relationship between Ca^{2+}-regulating proteins and μ-calpain in order to understand how their interactions affect pork quality.

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